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EXPERIMENTAL CELL RESEARCH

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Experimental Cell Research **13**, *1–10 (1957)*

EFFETS DES RAYONS U.V. SUR *ACETABULARIA MEDITERRANEA*[1]

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Reçu le 30 novembre 1956

Dans un travail précédent [1] nous avons étudié les effets des rayons X sur l'algue uninucléée *Acetabularia mediterranea* dans le but de mettre en évidence le rôle respectif du noyau et du cytoplasme dans la production des lésions.

Nous avions notamment observé des effets du noyau sur le cytoplasme et l'inhibition tardive de la morphogénèse. Comme les effets des rayons X et U.V. semblent être très différents en ce qui concerne la biosynthèse des protéines (synthèse induite notamment chez les microorganismes) il nous a paru opportun d'étudier l'effet des U.V. sur des algues entières ou sur des fragments d'algues. Un certain nombre d'observations avaient déjà été effectuées par J. Brachet [2] sur des fragments nucléés et anucléés. Comme nous l'avons dit précédemment, un travail de Hämmerling [3] et un autre plus récent de Six [10] ayant trait au même type d'observations ont été publiés il y a quelques mois.

MATÉRIEL ET MÉTHODES EXPÉRIMENTALES

Les algues utilisées proviennent de la culture faite au laboratoire dans les conditions décrites par Brachet, Chantrenne et Vanderhaeghe [4].

L'irradiation des algues est effectuée à l'aide d'une lampe mineralight non filtrée, les algues reposant au fond d'une boîte de Petri. L'intensité utilisée a été déterminée à l'aide d'un dosimètre de Latarjet [7].

Dans le cas où seules les tiges ou les rhizoïdes d'algues entières ont été irradiés, la partie non exposée (soit toute la tige, soit uniquement le rhizoïde) était protégée par une mince feuille d'aluminium. Les témoins de certaines de ces expériences avaient été irradiés avec une protection complète.

Comme dans le travail précédent, nous avons observé indépendamment

[1] Travail effectué par le Laboratoire de recherches pour la Protection des Populations civiles.

de la survie, la croissance des algues entières ou la régénération des fragments, et enfin la morphogénèse (formation de chapeaux).

D'autre part, nous avons noté, *pour une même algue*, l'évolution de la tige indépendamment de celle du rhizoïde car il arrive fréquemment que la première se cytolyse alors que le second reste intact et est même l'origine d'une nouvelle algue.

RÉSULTATS

Survie des algues

Comme on le voit dans la figure 1 (*A*, *B*), $8 \cdot 10^3$ ergs/mm^2 suffisent pour cytolyser complètement plus de 20 % des tiges d'*algues irradiées in toto* alors qu'il faut atteindre $24 \cdot 10^3$ r pour observer un effet appréciable sur les rhizoïdes des mêmes algues.

L'irradiation de *fragments* montre que pour les U.V., de même que pour les rayons X, la survie des fragments anucléés est davantage affectée (tableau I).

Dose $\times 10^3$ ergs/mm	% de survivants après 10 jours par rapport aux témoins	
	Nucléés	Anucléés
8	100	94
13	92	78
24	24	0

Si l'on examine la survie des parties d'algues entières dont la tige ou le rhizoïde ont été protégés au cours de l'irradiation (fig. 1, *C* à *F*) on constate qu'une forte irradiation de la tige (*E*) affecte la survie de la partie non irradiée.

Si on compare enfin la survie des tiges des algues entières irradiées *in toto* à celle des algues dont les tiges seules ont été irradiées, on constate que la présence d'un rhizoïde intact favorise la survie de la tige (fig. 1, *B* et *F*). Dans le cas de l'irradiation des rhizoïdes, le phénomène paraît moins évident (fig. 1, *A* et *C*).

Croissance ou régénération

Les mêmes critères de croissance ou de régénération ont été utilisés que dans le travail précédent.

Algues entières irradiées in toto.—Dans le cas des rayons U.V., on observe

TABLEAU I

	Survie	Croissance	Morphogénèse
X	Plus grande sensibilité des tiges.	Algues entières qui survivent croissent mieux que témoins. Fragments nucléés : régénération retardée, mais quantitativement égale. Fragments anucléés : régénération du même ordre que dans les témoins chez les algues jeunes, accélérée dans les algues âgées.	Inhibition de la formation des chapeaux dans les algues entières – mais effet tardif. Stimulation de la morphogénèse dans les fragments anucléés irradiés.
U.V.	Plus grande sensibilité des tiges. Effet favorable de la présence d'un rhizoïde sain sur la tige irradiée. Effet défavorable de la partie fortement irradiée sur la partie non irradiée.	Pas de différence dans la croissance des survivants ni pour les tiges, ni pour les rhizoïdes, des algues irradiées *in toto* (X). Pas de différence dans la croissance des fragments nucléés et anucléés. Stimulation précoce de la croissance des rhizoïdes ou des tiges des algues dont l'autre partie a été irradiée.	Inhibition de la formation des chapeaux dans les algues entières irradiées *in toto*, ou dont seule la tige est irradiée. Stimulation possible par irradiation des rhizoïdes d'algues dont la tige est protégée. Inhibition de la morphogénèse dans les fragments anucléés.

des résultats différents de ceux obtenus au moyen des rayons X. Dans ce dernier cas, les survivants étaient tous capables de croître; par contre nous observons ici que pour les fortes doses d'irradiations ($>8 \cdot 10^3$ ergs/mm^2) la croissance des survivants est sensiblement affectée (fig. 2, *A* et *B*, comparée à la fig. 1, *A* et *B*).

Algues entières partiellement irradiées (fig. 2, *E* à *H*).—Pas plus que dans le cas des rayons X, on n'observe d'effet favorable du rhizoïde sain sur la croissance de la tige irradiée (fig. 2, *B* et *H*) sauf peut-être pour la dose la plus forte ($24 \cdot 10^3$ ergs/mm^2). Par contre la présence d'un cytoplasme non irradié favorise considérablement la croissance du rhizoïde (fig. 2, *A* et *G*) (effet paradoxal pour 2, *G*).

Enfin lorsque l'on examine les effets de l'irradiation U.V. sur des *frag-*

ments nucléés et anucléés, on constate que l'irradiation diminue légèrement le pouvoir de régénération des algues qui ont survécu; l'effet est un peu plus marqué sur les fragments anucléés que sur les autres (fig. 2, *C* et *D*).

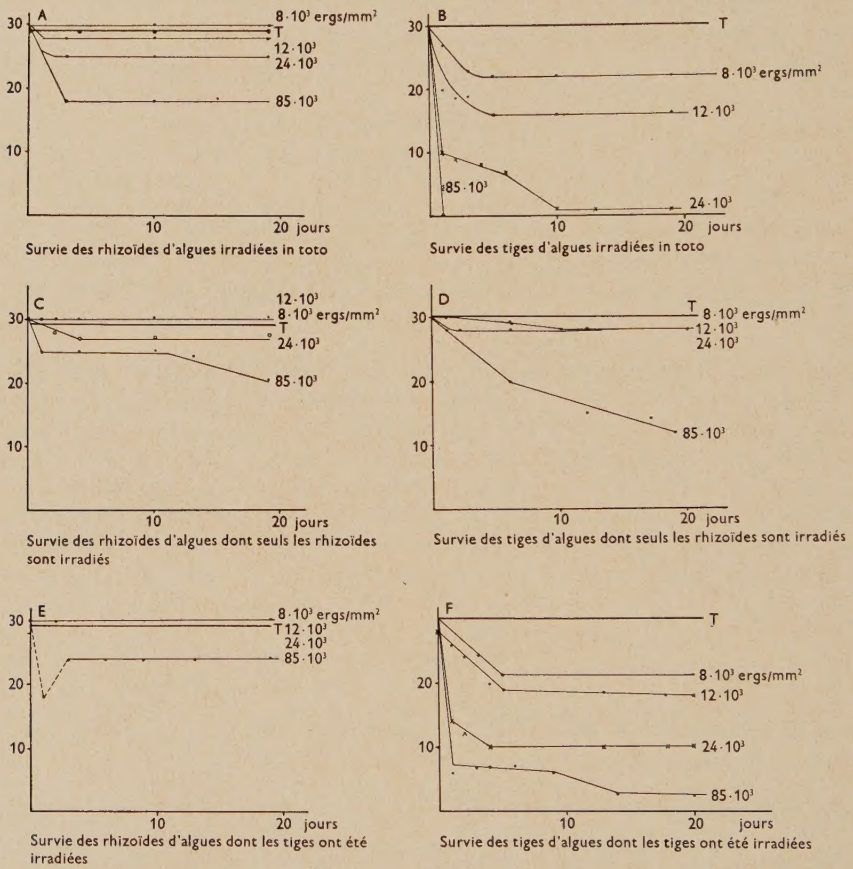

Fig. 1. Effets de l'irradiation U.V. sur la survie d'*Acetabularia.* Abscisses : nombre d'algues. Doses en ergs/mm².

Morphogénèse

La fig. 3 *B* montre que, comme dans l'irradiation par les rayons X, *les algues entières irradiées* forment moins de chapeaux que les témoins; elles en forment d'autant moins que la dose administrée est plus importante; en outre, leur apparition s'en trouve retardée.

L'irradiation d'*une partie de l'algue entière* donne des résultats inattendus. Lorsqu'on irradie fortement le rhizoïde ($53 \cdot 10^3$ ergs/mm²) on obtient une

stimulation importante de la morphogénèse (fig. 3 *A*); quand l'irradiation est plus faible ($24 \cdot 10^3$ ergs/mm²) la morphogénèse commence normalement mais, après une dizaine de jours, elle s'arrête; une dose encore moindre ($12 \cdot 10^3$ ergs/mm²) n'affecte pas de manière sensible la morphogé-

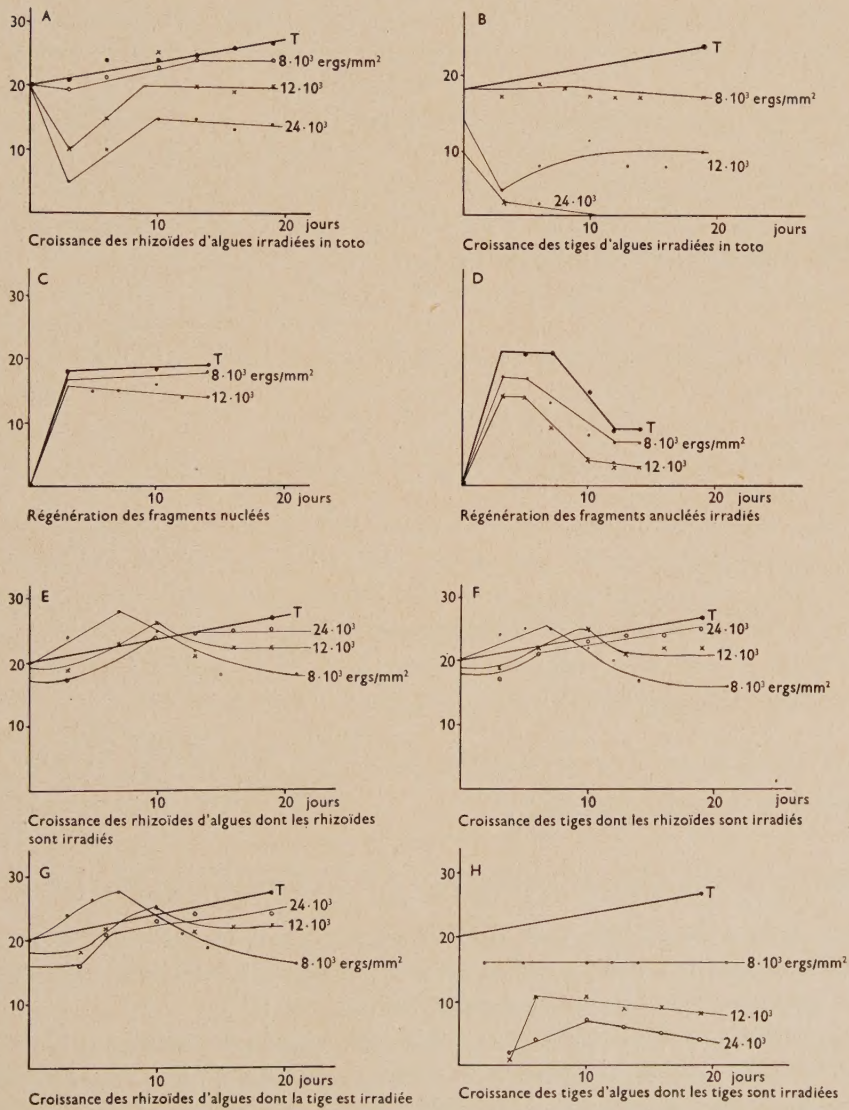

Fig. 2. Effets de l'irradiation U.V. sur la croissance et la régénération d'*Acetabularia*.

nèse (fig. 3, *A*). L'irradiation des *tiges*, par contre, inhibe la morphogénèse (fig. 3, *B*).

Si on irradie des *tiges isolées*, on observe également une forte diminution de la morphogénèse (fig. 3, *D*).

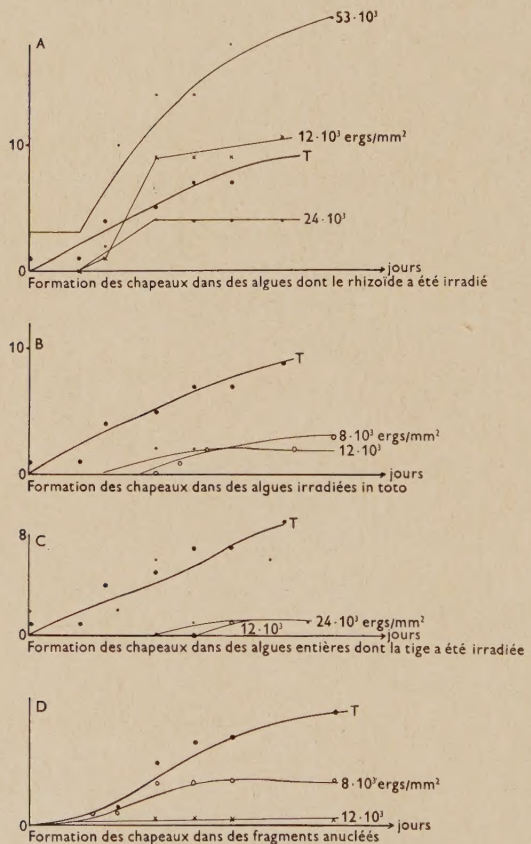

Fig. 3. Effets de l'irradiation U.V. sur la morphogénèse d'*Acetabularia*. Abscisses : nombre d'algues. Doses en ergs/mm².

Observations des coupes histologiques d'Acetabularia *irradiées par U.V.*

Nous avons étudié les effets de l'irradiation U.V. sur des algues entières, à différents temps après l'irradiation (0 h., 6 h., 48 h.) ainsi que les effets de l'irradiation du cytoplasme seul sur le noyau.

Irradiation des algues entières (22.000 ergs, algues de 2 mois ½).—L'irradiation a un *effet immédiat* à cette dose, bien que la plupart des algues survivent. Le noyau acquiert un aspect caractéristique : le nucléole garde sa structure boudinée, mais les vacuoles centrales s'élargissent, la croûte qui les entoure

devient plus basophile ainsi que le suc nucléaire dans un certain nombre de cas. La basophilie du cytoplasme périnucléaire augmente très nettement aussi.

Six heures après l'irradiation, certains noyaux ont le même aspect qu'immédiatement après celle-ci. D'autres noyaux, au contraire, ont évolué : les boudins nucléolaires deviennent coalescents, les limites sont plus floues et le nucléole paraît moins basophile. Parfois le cytoplasme est encore très basophile, parfois il est devenu pauvre en ARN.

Quarante-huit heures après l'irradiation, les anomalies nucléolaires subsistent : coalescence, vacuolisation, mais la basophilie paraît normale.

Irradiation du cytoplasme seul (20.000 ergs).—Des anomalies nucléaires se produisent aussi dans ce cas : elles sont un peu différentes. Il y a peu de vacuolisation, mais augmentation de la basophilie et de la coalescence des boudins, dont les contours sont flous. Le suc nucléaire est très basophile, comme le cytoplasme d'ailleurs.

Six heures après l'irradiation, l'effet se maintient et le nucléole tend à se vacuoliser.

DISCUSSIONS DES RÉSULTATS

Les *Acetabularia* constituent un matériel de choix pour l'étude des effets des radiations sur le noyau et sur le cytoplasme. On peut, en effet, soumettre à l'irradiation soit des fragments nucléés et anucléés, soit une partie seulement de l'algue restée entière : le rhizoïde, qui contient le noyau, et la tige, uniquement cytoplasmique. (On peut, en général, assimiler le rhizoïde au noyau, car le cytoplasme qui entoure ce dernier est négligeable par rapport au reste du cytoplasme de l'algue.)

Les principaux résultats que nous avons obtenus sont résumés dans le tableau I.

Le fait le plus frappant qui ressort de l'étude de la survie des algues irradiées est que le cytoplasme paraît un peu plus sensible aux radiations que le noyau. Ce fait s'observe non seulement lorsqu'on irradie des fragments nucléés et anucléés, mais aussi lorsqu'on irradie l'algue entière, totalement ou partiellement. Ce même résultat a été observé par Hämmerling [6] sur *Acetabularia* et par Mazia et Hirschfield [8], puis par Skreb [11] sur les Amibes. On ne peut toutefois pas exclure, dans le cas de l'algue, le fait que la partie nucléée est plus opaque aux irradiations.

Nous observons, en outre, que la présence d'un rhizoïde sain est favorable à la survie du cytoplasme irradié par l'U.V. Inversément, on peut constater

des effets réciproques du noyau irradié sur le cytoplasme sain et du cytoplasme irradié sur le noyau sain. De tels effets ont été rapportés aussi par Duryée [4] dans le cas des oocytes de grenouille et par Ord et Danielli [9] dans celui des Amibes. En ce qui concerne plus spécialement les *Acetabularia*, nous avons pu mettre en évidence, par voie cytochimique, des anomalies nucléaires particulièrement nettes tant lorsque le rhizoïde est soumis à l'irradiation U.V., que lorsque la tige seule est irradiée.

Si on examine, non plus la survie, mais la *croissance* des algues entières et la *régénération* des fragments, on trouve des différences assez frappantes entre les effets produits par l'irradiation X et ceux produits par l'U.V. Ainsi, alors que l'irradiation X ne semble atteindre que les algues qui ne croissent pas, l'U.V. affecte à la fois la survie et la croissance des algues qui ont survécu.

Notons aussi que les fragments nucléés et anucléés irradiés par les X régénèrent, aux doses utilisées, soit dans les mêmes proportions (expér. X1) soit mieux que les témoins (expér. X2); au contraire, les fragments irradiés par l'U.V. montrent une inhibition de la régénération d'autant plus importante que la dose administrée a été plus forte. C'est ce qu'observe également Hämmerling [6] pour l'U.V.

Nous avons pu noter encore que l'irradiation par l'U.V. du rhizoïde seulement de l'algue intacte, favorise la croissance de celle-ci, du moins pendant une période initiale; de même, l'énucléation favorise la croissance du cytoplasme.

Enfin, l'étude de la *morphogénèse* des algues irradiées n'a fait que confirmer et accentuer les observations faites à propos de la croissance. Alors que la morphogénèse est inhibée par l'irradiation U.V. des tiges, qu'elles soient nucléées ou non, elle est au contraire favorisée par l'irradiation du rhizoïde seul. L'irradiation par les X des fragments anucléés hâte et favorise l'apparition des chapeaux dans ces fragments.

Il existe relativement peu de phénomènes biologiques qui répondent aussi différemment à l'action des rayons X et U.V. Il en est au moins un autre, c'est celui de la synthèse des enzymes adaptatifs chez les microorganismes qui est relativement sensible aux rayons U.V. mais elle l'est généralement fort peu aux X (voir références dans [5]). Dans le cas d'*Acetabularia*, les rayons X semblent accentuer les effets de l'énucléation sur la tige et ils respectent les facteurs cytoplasmiques nécessaires à la morphogénèse. L'U.V., au contraire, atteint plus spécialement ces facteurs ainsi que ceux qui répondent de la croissance. Cette différence doit peut-être être rapprochée du fait que l'acide ribonucléique est moins sensible aux rayons X que l'acide désoxy-

ribonucléique, alors que les deux types d'acides nucléiques sont affectés par les U.V.

L'inhibition de la morphogénèse dans les fragments privés de noyau, par les U.V., qui ne se produit pas après l'irradiation X, permet de penser que l'un des constituants au moins des « facteurs morphogénétiques » du cytoplasme est l'ARN. Cette interprétation paraît d'autant plus justifiée que Six [10] vient de montrer que l'inhibition de la morphogénèse est plus importante à 254 mμ qu'aux longueurs d'onde plus élevées. Ainsi s'expliquerait aussi l'effet favorable, sur la morphogénèse, obtenu par l'irradiation du rhizoïde seul : elle favoriserait la synthèse de l'ARN cytoplasmique aux dépens de celui du noyau, comme lors de l'énucléation.

RÉSUMÉ

Le cytoplasme de l'algue *Acetabularia mediterranea* paraît plus sensible à l'irradiation UV que le noyau. L'irradiation affecte davantage, en effet, la survie et la régénération des fragments anucléés que celles des fragments nucléés de l'algue. De plus, l'irradiation du cytoplasme seul de l'algue (restée entière) est presqu'aussi défavorable que l'irradiation totale de celle-ci. Par contre, l'irradiation de la partie nucléée de l'algue est peu ressentie et peut même, dans certains cas, provoquer une stimulation précoce de la croissance et de la morphogénèse.

Des observations cytologiques ont montré que des altérations nucléaires se produisent aussitôt après l'irradiation, que ce soit la partie nucléée ou le cytoplasme seul qui ait été irradié.

SUMMARY

The cytoplasm of *Acetabularia mediterranea* appears to be more sensitive to UV irradiation than the nucleus, as demonstrated by a decreased survival and regeneration of anucleate fragments as compared with the nucleate ones.

Irradiation of the cytoplasm only of a whole cell has the same effect as irradiation of the entire cell. When only the rhizoïd (which contains the nucleus) is irradiated, there is less injury than when the whole cell or only the cytoplasm is irradiated. Irradiation of the rhizoïd only can even cause a precocious stimulation of growth and morphogenesis.

Cytological observations have demonstrated an immediate nuclear damage, even though the nucleus was not within the irradiated area.

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STUDIES ON THE MECHANISM OF SYNCHRONOUS CELL DIVISION IN *TETRAHYMENA PYRIFORMIS*

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In a preceding paper it has been shown how the mean cell size changes in normal and heat-treated mass cultures of *Tetrahymena pyriformis* GL under standardized conditions [13]. In the course of eight generations of exponential growth (19 hours) the mean size is reduced to about 50 per cent. After eight hours of intermittent temperature treatment, however, the cell size is three- to fourfold of that prior to treatment. These observations suggested further analysis of the overall growth rate (i.e. mass increase) and reproduction rate (as expressed in the generation time) under the conditions mentioned [18].

Aside from these rate studies at constant temperature, the velocity of the restoration process(es) after heat treatment will be analysed as a function of temperature. It was earlier proposed that during the constant delay before onset of the first synchronous cell division in a culture which is released from the standard temperature treatment, processes might occur which are essential also in preparing the normal cell for division [17].

Kinetic studies by Johnson and Lewin on the reproductive rate of *Escherichia coli* [8] stimulated similar analysis in single cell cultures [20] and synchronized mass cultures of *Tetrahymena*. The experimental results so far obtained will be analysed in the light of the theory of absolute reaction rates [7]. It must be kept in mind, however, that such complicated processes as cell growth and division may depend on a multitude of individual, consecutive and interlinked reactions. Any model in its first approximation might suffer from oversimplification. It will, however, provide a rational quantitative basis for the analysis of the data.

MATERIAL AND METHOD

Organism.—Tetrahymena pyriformis GL is grown in 2 per cent (v/w) peptone media at temperatures optimal for division (28–29°C). The heat treatment lasts 7.5 hours. In the course of this time the temperature of the culture medium is rhythmi-

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cally changed at half hour intervals (28.5°⇌33.9°C). Growth and culture conditions were given in detail earlier [13, 18].

Cell counts and cell size.—A Sedgewick–Rafter counting chamber and Whipple micrometer was used for direct counts [14]. The cell size was calculated from direct measurements of both cell diameters on photographs of fixed specimens [13]. In Fig. 1 each point on which the curves V_n and V_h are based was calculated in the following way: from the measurement of 100 cells on the photographs the average cell size was determined and this value multiplied with the corresponding cell number per ml.

KINETIC STUDIES

Growth and cell division in mass cultures.—In a series of four parallel experiments the overall increase in population density was followed over eight generations under optimum conditions for cell division. If at the same time the average cell size is determined and the mass increase calculated, a slightly, but definitely different slope is obtained. This may be illustrated by Fig. 1. The generation time (the time required to double the population number) under normal condition is 138 minutes, as it is given by the slope of the curve

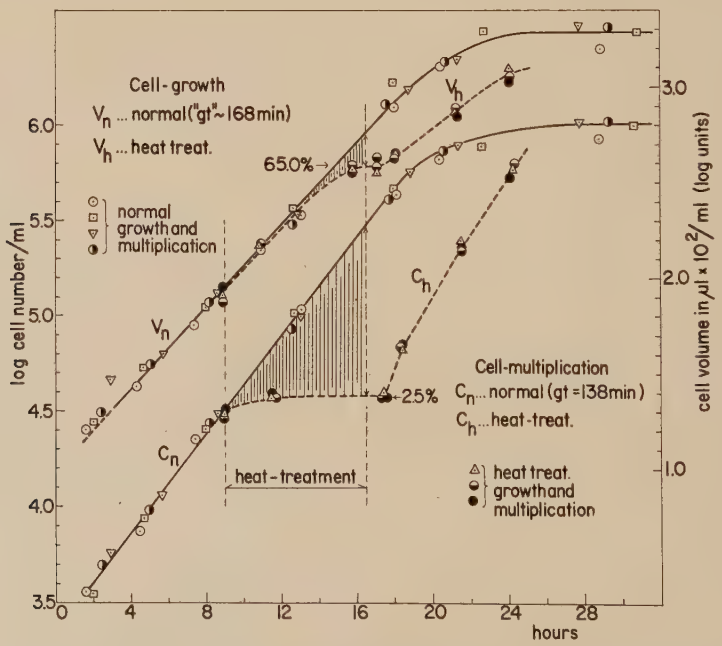

Fig. 1. Growth and multiplication in normal and heat-treated mass cultures. The logarithm of cell number per ml (on the left ordinate) and total cell volume per ml (on the right ordinate) is plotted against the incubation time in hours on the abscissa.

C_n. The "generation time" for the doubling of the total cell mass is 168 minutes. However, this picture is drastically changed if such a culture, grown to a population density of about 3×10^4 cells per ml under normal conditions, is exposed to the standard heat-treatment. The broken lines C_h and V_h in Fig. 1 represent the population count and mass increase of three such experiments, respectively. While the treatment exerts its full effect on cell division already in its early part (curve C_h), total mass increase is apparently not effected at all within the first four hours (curve V_h). The parallel vertical lines illustrate this differential effect on growth and cell division in a mass culture. At the end of the treatment, cell multiplication is reduced to 2.5 per cent and mass increase to 65 per cent of what we would have found in a normal, untreated culture. We shall come back to these curves in the discussion.

Relative rate of multiplication in normal and synchronously dividing mass cultures.—We have suggested that every time that cells are exposed to a temperature shock in the course of the treatment some metabolic steps towards cell division are inhibited [17]. These processes might occur, however, in the time span between the last temperature shock and the first synchronous division to restore the chemical situation which releases cell division. We shall refer to this induced lag as the recovery or restoration period. It was found to be surprisingly constant at specific temperatures and independent of the culture medium in which the cells were grown [18].

Several experiments were carried out in which samples of heat-treated cultures were transferred to various temperatures between 10° and 33°C and the first synchronous division maximum was observed. The results are shown in Fig. 2. The relative rate of recovery is given as a function of the absolute temperature (symbol ●). To facilitate comparison with the data by other authors, the maximum rate is arbitrarily taken as 100. Symbol (◑) shows the relative rate of multiplication in single cell cultures of *Tetrahymena pyriformis* GL [20]. The results as represented by symbol (⊗) were calculated from data by Phelps on the multiplication rate of *Tetrahymena pyriformis* TP as a function of temperature between 9.6°C and 28.6°C [12]. Both curves are fitted by eye to the experimental points. Symbol (○) shows the relative rate of multiplication of *Escherichia coli* [8]. The theoretical curve around which the experimental points scatter was calculated by Johnson, Eyring and Polissar with the help of an equation which was derived on the basis of the following consideration: the net rate of multiplication under the conditions described can be quantitatively accounted for, to a large extent, on the basis of two reactions of a single enzyme system. The

rate of this enzyme reaction at different temperatures, which is supposed to express itself in the slope of the logarithmic growth curve, is governed by (*a*) the activation energy of the rate limiting reaction, and (*b*) an equilibrium between active and inactive forms of the catalyst. This formula is [7]:

$$G = \frac{cTe^{-\Delta H^{\mp}/RT}}{1 + e^{\Delta S/R} e^{-\Delta H/RT}},$$

where G stands for the relative rate of multiplication. In the calculation of G for *Escherichia coli* the following figures are cited by Johnson, Eyring and Polissar [7]: the heat (ΔH) and entropy (ΔS) of the denaturation equilibrium are found to be quite high (150,000 for ΔH and 476.46 for ΔS), while the activation energy of the rate-limiting reaction ($\Delta H^{\mp} = 15{,}000$) is typical for enzyme-catalysed processes [7]. These figures check well with the kinetic data as calculated by Thormar on the relative rate of multiplication in *Tetrahymena pyriformis* GL [20].

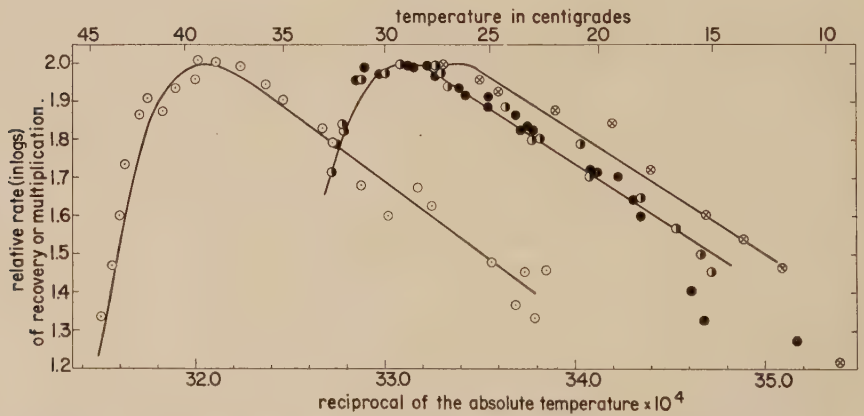

Fig. 2. Relative rate of recovery and multiplication as a function of temperature. ● Relative rate of recovery in a synchronized mass culture of *Tetrahymena pyriformis* GL. ◑ Relative rate of multiplication in single cell cultures of *Tetrahymena pyriformis* GL [20]. ⊗ Relative rate of multiplication in mass cultures of *Tetrahymena pyriformis* TP [12]. ○ Relative rate of multiplication of *E. coli* [8].

DISCUSSION

Rate of growth and multiplication in mass cultures.—A similar difference in the rates for the normal increase of total cell mass and total cell number in the course of several generations of exponential multiplication (Fig. 1) was also observed in bacterial growth. Hershey determined the total nitrogen together with the cell number (viable counts) of an exponentially growing

culture of *E. coli* [3]. The generation time was found to be about 11 minutes, while the time required to double the cell mass was 23 minutes. This phenomenon is necessarily reflected in the size of the individual cell. Or, as Hinshelwood clearly pointed out: The cell size depends upon the ratio of the rate of growth to the rate of division. This ratio is not constant in general throughout the exponential growth phase [4]. Such experiments might suggest that mass increase (i.e. growth) and multiplication are governed by two separate processes functioning simultaneously. This very idea was already put forward by Swann as a result of extensive studies on the effect of inhibitors on the early cleavage stages of fertilized sea urchin eggs [19].

Relative rate of multiplication in normal and synchronously dividing mass cultures.—Fig. 2 shows that the data from the experiments on the rate of recovery in synchronized cultures given by symbol (●) and for the rate of multiplication in normal cultures of *Tetrahymena pyriformis* GL (symbol ◑) can be considered identical. This conclusion leads to the suggestion that the cell system(s) affected by heat shocks might be identical with the rate-limiting process determining the generation time in normal multiplication.

A comparison of the shapes and slopes of the curves for the relative rate of multiplication in *E. coli* (symbol ○, Fig. 2) and *Tetrahymena pyriformis* GL, TP (symbols ◑, ⊗) gives the impression of similar rate processes, yet with different maxima. The similarity of the rate curves for multiplication in the bacterial and protozoan cultures shown suggest an explanation for the data obtained for *Tetrahymena* in the same way as Johnson and Lewin interpreted their results of *E. coli* [8, 7]. These results (symbol ○, Fig. 2) agree well with the theoretical curve calculated with help of the equation presented. The basic assumption from which this equation was derived—the reversible denaturation of the enzyme in question—appears to be well established by kinetic studies on single enzyme systems *in vitro*, for example the trypsin inhibitor, a pure crystallized protein, isolated by Kunitz from soybeans [9] and luciferase isolated by Chase from the crustacean *Cypridina* [2]. Anson and Mirsky have stated that such a reversible protein denaturation might play an important role also in ordinary cellular processes [1]. The quantitative significance of the denaturation equilibrium as a controlling factor in physiological processes *in vivo* was first demonstrated in connection with bacterial luminescence [6].

For a successful analysis of the data on *Tetrahymena* it was necessary to gain some quantitative information not only on the response of mass cultures to a single heat shock or to a sequence of intermittent heat treatments, but

also on the "sensitivity" of individual cells in different parts of their reproductive cycle. Preliminary observations left us with the impression that prior to division the cells pass into a "most sensitive" phase [18]. Conclusive experimental evidence, however, was established by Thormar [20]. His

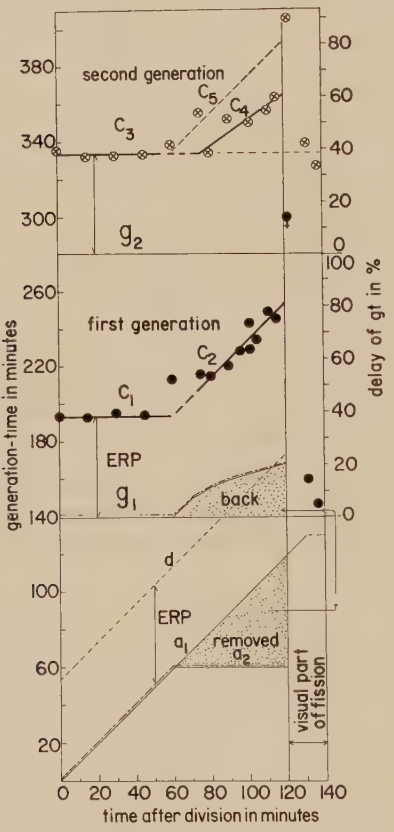

Fig. 3. The length of the generation times as a function of the age of the cell, when a heat shock was administered. (Experimental points from a paper to be published by H. Thormar [20]). Further explanations in the text.

results are presented in Fig. 3. At various known intervals after the completion of cytoplasmic fission, single cells were exposed to a single temperature shock (15 minutes at 34°C) and returned to 28.5°C. The time after fission, when the heat shock was applied, is given on the abscissa. On the ordinate the time span between the completion of successive divisions (i.e. the generation time) is recorded. The period of heat exposure is subtracted in all cases. The normal generation time is 140 minutes (solid line g_1). However, if cells at different positions in their reproductive cycle are exposed to a single shock (line a_1), the following cell division is delayed, as given by symbol

(●). The degree of delay can be read from the interpreted curves c_1 and c_2. The second division (second generation after treatment) which should occur normally at g_2 is similarly delayed (symbol ⊗). Before making any attempt to interpret these data we must turn to some general aspects of the problem.

On the occasion of synchronization of cell division in pneumococcal cultures, Hotchkiss advanced the idea that biochemical processes of cell growth and division would be disrupted by temperature changes. "Certain of the enzyme systems", he says, "should now be less able than others to transfer an amount of substrate equal to that supplied them, and there would then be a tendency for the metabolism to be selectively slowed or even temporarily halted at certain points" [5]. This theory was accepted and modified by Lark and Maaløe for the explanation of their data on synchronously dividing cells of *Salmonella typhimurium* [10]. We might specify this general hypothesis as forwarded by Hotchkiss in the light of the analysis of single enzyme systems, as carried out by Johnson, Eyring and Polissar [7].

As pointed out above, Johnson and Lewin assumed that the generation time of an exponentially growing culture of *E. coli* is proportional to the velocity of a limiting, enzyme catalysed reaction. If this assumption holds true also for the growth of *Tetrahymena* under similar conditions, there should occur or exist in the course of the life span of an individual cell (about 140 minutes at optimum incubation temperature for *Tetrahymena pyriformis* GL): (*a*) The synthesis of the hypothetic enzyme. (*b*) A supply of substrate for the reaction. (*c*) A denaturation equilibrium of this enzyme, dependent on the incubation temperature. (*d*) The enzyme reaction itself, which is highly temperature-dependent. The rate of this reaction should express itself in the slope of a growth curve in a mass culture. (*e*) The formation of the reaction product (RP), a certain amount of which is necessary for cell division. This assumption of a threshold concentration of RP is based on experiments, which led Swann to a reservoir hypothesis, explaining the control of cell division [19]. A similar idea of a threshold concentration of certain chemical substances required for cell division is stated by Hinshelwood [4].

We may now return to the discussion of the factors involved in the hypothetic enzyme reaction outlined above.

Supply of substrate for the reaction.—Johnson, Eyring and Polissar in their formulation of reactions in the temperature activity curve assumed the amount of substrate to be constant and without any influence on the reaction itself. The substrate concentration will therefore be considered so high, relative to that of the enzyme, that the enzyme is practically saturated.

The denaturation equilibrium of the enzyme.—In the experiments on which Fig. 3 is based, the height and length of the heat shocks to which the cells were exposed in different parts of their individual life cycle, were the same. Therefore a fixed proportion of the amount of the enzyme present in the individual cell was inactivated. This resulted in a constant denaturation equilibrium in the enzyme system in all cells. If the temperature shock to which the cell is exposed is of effective height and length, this denaturation equilibrium might well be in favour of the inactive form of the catalyst. Therefore, at the end of the heat shock we may have: $E_d \geqslant E_n$ (E_n stands for the active, E_d for the inactive form of the catalyst). After the lapse of a constant length of time, however, all the inactivated catalyst is activated and catalyses the reaction at normal rates, only delayed by the length of the heat shock plus the time span required to restore the catalytic activity of the enzyme (enzyme-restoration process, ERP).

The formation of the reaction product.—According to Swann [19] and Lark and Maaløe [10] we assume that a given concentration of the reaction product (RP) is required to initiate cell division. The velocity of its formation is dependent on the amount of the active form of the catalyst present. During exposure to heat shocks the amount of RP does not—in accordance with our assumption—significantly increase. Therefore the possibility remains that the amount of RP is unaltered or decreases, or that it is removed from the site of its formation for processes which are not (or not to the same extent) slowed down under the stress condition of heat exposure.

It is necessary now to consider the experimental data presented in Fig. 3 in the light of the postulated rate-limiting enzymatic process. The curves c_1 and c_2 give the delay of the first generation after exposure of cells of known age to a single heat shock. Let us first inspect the curve c_1. If a cell—up to an age of about 60 minutes (curve a_1)—is exposed to a single heat shock, its following division will be delayed by a constant time span of 54 minutes. This is read as the difference $c_1 - g_1$. Considering the enzymatic process limiting the generation time, the situation may be as follows: up to the age of 60 minutes the enzymatic process (EP) builds up RP, indicated by the broken line above a_1. After the end of the heat shock the restoration of the active form of the enzyme takes place (ERP). When the activity of the enzyme is restored the synthesis of RP is resumed with normal rate, and the generation time will be completed after the constant delay ERP. In other words: irrespective of the age of the cell up to 60 minutes, the generation time is increased by about 54 minutes. This is the constant time span which might be necessary for the re-activation of about 50 per cent of the catalyst

which was inactivated by heat. This recovery of the enzyme activity might have a slightly negative activation energy, as it was found in the luciferase system [7]. One would expect therefore that ERP occurs spontaneously under optimum conditions. That this is not the case, however, can readily be understood on basis of the theory of absolute reaction rates [7].

Curve c_2 in Fig. 3 shows a marked increase of the normal generation time g_1 after heat exposure in the latter part of the reproductive cycle. This can obviously be due to a gradual decrease of the rate of the enzyme recovery (ERP) after heat exposure or a removal (inactivation) of the RP. It appears to be very unlikely that the enzyme system should become "more sensitive" in the latter part of the cycle, i.e. the recovery of the active form of the catalyst should be progressively inhibited. The other alternative is, as already pointed out, that a given amount of RP is inactivated or removed from the pool for processes less affected by the heat shocks. This assumption may be supported by cell size studies of normal and synchronously dividing cell populations. These studies show definitely that bigger cells, representing the latter part of the reproductive cycle, grow more on a percentage basis than smaller cells representing the earlier part of the cycle [13].

Looking at curve d (Fig. 3) in the period 60–120 minutes after division we have the time position of the cell when the enzyme is reactivated after the heat shock. The difference $(c_2 - d)$ is surprisingly constant. Therefore $g_1 - (c_2 - d)$ yields a_2, which indicates that the RP pool, normally increasing in time, is levelled to that amount which is present in cells of about 60 minutes old. This levelling-effect on the RP pool may be considered important in the mechanism of synchronisation of cell division in a mass culture. The effect of a single heat shock on a mass culture consists in a synchronous burst of cell division of 30–35 per cent at the best [21, 15]. This result agrees well with the theoretical value of 38 per cent. This figure represents the fraction of cells within the time span 60–120 minutes after division (indicated by a_2 in Fig. 3) on a percentage basis of the cell number in the whole reproductive cycle. In this calculation the fact must be considered, however, that in an exponentially growing mass culture there is a gradient of cell number at different ages, young cells being more numerous than old ones. This is due to the fact that two young cells are produced by one old cell through the fission process. This phenomenon we may refer to as the "age gradient". Details are given elsewhere [16].

In the second generation after heat exposure we have again the constant delay (ERP) in the first part of the cycle. This means that the reproductive cycle is normal with a generation time $(c_3 - c_1)$ of 140 minutes. If the latter

part of the cycle would be normal we should expect the division to be completed at c_5. This is apparently not the case. The cycle is already completed earlier (c_4), indicating a regulatory mechanism of partial re-filling of the RP pool, which was removed during the heat shock. The extent of this suggested feeding-back mechanism is indicated by the arrows at g_1.

Enzyme synthesis.—If cells at different positions in the reproductive cycle are exposed to a single temperature shock, the following cell division will be delayed. The length of this delay depends on the age of the cell (Fig. 3, curves c_1, c_2). There is some experimental evidence (Fig. 3) that in the latter $\frac{2}{3}$ of the visual fission process there is not such a delay, at least much less than the time taken for ERP. This indicates that the mechanism proposed above is not at work in this phase, but the enzyme in question may be activated there, in preparation for the next cycle. This is suggested from the observation (Fig. 3) that a cell in the latter part of fission, which is almost not at all delayed in the first generation after heat exposure, shows in the second generation a delay of about 54 minutes. This is the characteristic time span for ERP of cells after fission in the first generation.

As will be suggested later, the enzyme synthesis in question is retarded to the same extent as the growth processes in the course of the intermittent heat treatment of a mass culture. One might therefore think that the enzyme is normally synthesized in the time span between two divisions and activated in the latter part of cell division. After its activation, the enzyme builds up the RP pool for the following division. In other words, synthesis of enzyme in the inactive state occurs continuously throughout the preceding cell cycle. This is a view which may have some support from Swann's reservoir hypothesis [19].

Quantitative aspects of enzyme concentration and RP-pool size in normal and synchronized mass cultures.—As shown, the ERP takes about 54 minutes and the time span from the last shock to the first division peak in a mass culture is 75 minutes. The actual synthetic process lasts therefore 21 minutes in the treated culture, while in the normal reproductive cycle it takes 130 minutes (the normal generation time minus 50 per cent of the time taken for the visual part of fission (15–20 minutes [21], a stage which was chosen for the characterization of the synchronous division peak). According to the assumptions previously made, the velocity of the synthetic process is proportional to the enzyme concentration. The rate of this enzyme reaction, on the other hand, is supposed to express itself in the length of the generation time (see above). It is evident, therefore, that the enzyme concentration in the synchronized cells should increase 130/21, that is about 6.2 times. This

must be compared with the situation in the untreated control cultures in the course of the time span corresponding to the heat treatment. The generation time for normal exponential multiplication was found to be 138–140 minutes (Fig. 1). Since the treatment lasts 450 minutes, the normal population goes through 3.07 generations. The corresponding increase in cell number is $2^{3.07}$, i.e. 9.2 times. Comparison of this figure with that obtained for treated cells shows that the enzyme synthesis in a synchronized mass culture is reduced to 67 per cent of the control. This figure is surprisingly close to that of 65 per cent of mass increase during treatment (see Fig. 1).

With relation to the pool size, on the basis of earlier arguments, we assume that the RP is formed at the same rate throughout the cycle. In Fig. 3, curve a_1 may represent not only the time position of the cell in its reproductive cycle, but at the same time may indicate the size of the RP pool as a function of time. This size is given in a mass culture by the area between curve a_1 (Fig. 3) and the abscissa in the limits 0–120, times the "age gradient" [16]. Similarily, we can calculate the RP pool size prior to division in a synchronized culture. If all cells would divide at the same time, the concentration of RP would be in all cells equal, corresponding to the amount present in a cell just entering the visual part of fission (i.e. 120 minutes after last division).

The results of these estimates are presented in Table I.

TABLE I

Estimation of the hypothetical relative concentration of the activated enzyme and relative pool size of the reaction product (RP) at different growth stages as expressed per average cell. Further explanation in the text.

Type of culture	Concentration of enzyme in active state	RP-pool size
Normal culture. . . .	1	1.0
After heat treatment .	0	0.0
Prior to division . . .	6	2.4

CONCLUSIONS

Based on the considerations set forth above, the following scheme of synchronization mechanism is suggested:

(1) Processes of cell growth and processes leading to cell division may function simultaneously with their own characteristic rates (Fig. 1) indicating separate, but interdependent (Fig. 3) processes.

(2) Intermittent heat treatment disrupts existing equilibria in the metabolism of these two sets of processes to different degrees, with the net result that after the treatment growth is reduced to 65 per cent of the normal values. Cell division is suppressed almost completely (making allowance for the cells in division to complete fission).

(3) In the course of the intermittent heat treatment the RP pool, a given threshold concentration of which is required to induce the fission process, is completely emptied. This phenomenon occurs already in part after one heat shock (Fig. 3, curve a_2), resulting in a partial synchronization effect with a division index of 30–40 per cent (*first phasing effect*).

(4) The enzyme, inactivated by the elevated temperature cycles, is reactivated to about 50 per cent in 54 minutes.

(5) In the course of the treatment, enzyme is formed in the inactive state at a rate (67 per cent of the control) which is characteristic for mass increase during treatment (*second phasing effect*).

(6) The synchronous division steps after release from heat treatment constitute a back regulation of the disrupted systems to equilibria and rates normally found in an untreated cell.

We are fully aware that this picture of the possible control of the synchronization effect in particular and the mechanism of cell division in general may suffer from oversimplification. However, the hypothesis forwarded may stimulate further work in this field to identify the nature of the proposed mechanism.

SUMMARY

Experimental data on the synchronization of cell division in *Tetrahymena pyriformis* GL are analysed in kinetic respects. On this basis a scheme for a probable mechanism producing the synchronization effect is presented.

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THE CONTENT AND COMPOSITION OF NUCLEIC ACIDS IN NORMAL AND SYNCHRONOUSLY DIVIDING MASS CULTURES OF *TETRAHYMENA PYRIFORMIS*[1]

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A considerable body of evidence shows the relative stability of deoxyribonucleic acid (DNA) in cells of various tissues [5, 18]. This is also true for the content of DNA, though not for ribonucleic acid (RNA), in tissues undergoing drastic physiological and pathological changes [18]. It was proposed therefore to use the DNA content as a measure for cell number in adult tissues in which cell divisions are rare [5]. However, in a tissue with relatively many proliferating cells, such as a regenerating rat liver, the DNA content per nucleus is higher than normally found [5].

In the light of these findings one would expect no increase of the DNA content in cells the proliferation of which was impeded by the intermittent temperature treatment to induce synchronous cell division. Preliminary experiments indicated that this is definitely not the case. Instead, the DNA content per cell was found to increase fourfold in the course of the treatment [23]. Further studies showed different incorporation rates of P^{32} into the four nucleotides of RNA in different growth stages of *Tetrahymena* [12]. Therefore the present investigation was undertaken to elucidate the effect of the temperature treatment on the absolute amounts and relative composition of DNA and RNA on an average cellular basis.

MATERIAL AND METHODS

Organism.—*Tetrahymena pyriformis* GL was grown in peptone media as previously described [14]. As it was found necessary to grow larger quantities of cells for the quantitative estimation of the DNA bases, the final procedure adopted was therefore the following: 3 ml stock cultures were grown in test tubes. After three days of growth 1 ml of such a culture was transferred to 150 ml medium in 450 ml vessels as described [14]. After three days 3 ml aliquots were removed with Pasteur pipettes for the inoculation of six 2 l Fernbach culture vessels, each containing 1 l medium.

[1] This work is a part of a research program on the nucleic acid metabolism in *Tetrahymena*, carried out in the Fibiger Laboratory.

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After two days of incubation at the experimental temperature, the cultures were pooled in an incubator. This bath—100 × 40 × 10 cm in size—was made of brass, coated with the nontoxic resin "prodor glas" (Dansk-Prodor-Glas, Copenhagen). A glass plate was used as a lid. The bath was gently shaken around its minor axis with 22 strokes per minute to ensure proper aeration of the 15 mm layer of the culture medium. A plastic housing was built around the incubator, which was sterilized prior to every experiment with help of steam and a Letheray Germicidal Unit (Hanovia, Newark). Samples of 5 ml were removed from the experimental culture for cell counts as described [13] and for sterility tests by direct microscopical observation in phase contrast.

Estimation of the nucleic acids

Isolation of RNA.—The method followed was in principle the Schmidt–Thannhauser–Schneider procedure [19]. For each growth step under investigation, 1–2 liters of culture fluid were centrifuged in one liter centrifuge tubes of a 12 l centrifuge (International Serum Centrifuge, Model 13 L) at 90 *g* for 10 minutes. The sediment was removed with a pipette from the specially made funnel-shaped bottom of the centrifuge glass. The recovery was 90–95 per cent as calculated from direct cell counts prior to and after centrifugation. The concentrated cell suspension was transferred to 25 ml steel tubes of a International Centrifuge, Model PR-1, and centrifuged for 5 minutes at 3500 *g*. The clear supernatant was discarded and the residue homogenized with acetone in a Waring-Blendor for 2 minutes in the cold. Acetone powder was prepared according to Plesner [8], and 0.5–1.0 g was obtained for each growth step. The method of cultivation and collection of such quantities of synchronized cells will be described in detail by Plesner [9]. 200 mg dry acetone powder, used for each analysis, was freed from the acid-soluble compounds in a Potter–Elvehjem homogenizer with 4 portions of 5 ml ice-cold $HClO_4$ (5 per cent). The residue was washed twice with 4.5 ml alcohol (95 per cent) and the phospholipids were extracted with 3 portions of 4.5 ml alcohol–ether mixture (3:1). After digestion at 37°C for 24 hours with 4.5 ml 1 *N* KOH, the hydrolysates were neutralized with 6 *N* $HClO_4$ and 6.5 ml of ice-cold 5 per cent $HClO_4$ were added to precipitate the DNA and proteins. The supernatant, containing the nucleotides of RNA, was made up to 20 ml with water. One ml of this stock solution was neutralized with conc. KOH, evaporated and digested in 100 μl of 1 *N* HCl for one hour at 100°C. In this way, the purine bases together with the pyrimidine nucleotides of RNA were obtained, as described by Smith and Markham [17].

Isolation of DNA.—The DNA–protein precipitate, obtained as just described above, was dissolved in 5 ml of 0.5 *N* K_2CO_3. This solution was adjusted to pH 6 with 20 per cent CH_3COOH, and the DNA precipitated with two volumes of 96 per cent alcohol at + 3°C for three hours [15, 16]. The dried precipitate was hydrolysed in 150 μl of 72 per cent $HClO_4$ at 100°C for 1 hour and most of the acid was removed by destillation in vacuo.

Chromatography.—Filter paper Whatman no. 1, washed in 2 *N* CH_3COOH and distilled water, was used. Hydrolysates of RNA and DNA corresponding to 10 mg and 100 mg acetone powder, respectively, were pipetted on to the paper. Two-dimensional chromatograms were run with an isopropanol–HCl mixture in the first dimen-

sion [21] and a butanol–NH_3 mixture in the second dimension [7]. At the same time a blank sheet was run. The spots were located by Mineralight (Ultra-Violet-Products, Inc., Los Angeles). The compounds were identified by their R_f values and the absorption spectra of the eluates. The bases and nucleotides were eluted with 0.1 N and 0.01 N HCl, respectively.

Spectrophotometry.—A Beckman spectrophotometer, Model DU, was used. The wavelengths at which the concentrations of the purines and pyrimidines were measured and the corresponding millimolar extinction coefficients employed, are given by Wyatt [22].

EXPERIMENTS AND RESULTS

Growth steps chosen.—Six liter mass cultures of *Tetrahymena* were grown in the incubator for about 3 hours. At densities between 70–90 $\times 10^3$ cells per ml the first sample was removed, which served as a control for normal logarithmic growth (0 hours). Further samples were taken at the end of the temperature treatment after the application of seven heat shocks when no cells are in division (6.5 hours), and prior to (7.5 hours) and after (8.0 hours) the first synchronous cell division. These growth stages have the numbers 1 to 4 respectively in the corresponding sequence in Table I.

TABLE I

Dry weight and nucleic acid content per cell at different stages of normal and heat-treated population growth.

No. 1 refers to a normal mass culture (control). No. 2 represents a culture after application of seven heat shocks, No. 3 one hour later, prior to the first synchronous cell division. No. 4 represents a culture after the first synchronous division. The figures are average values from three parallel experiments for dry weight and DNA. The values for RNA are averages from two parallel experiments. The standard error for the estimation of the nucleic acids is as follows (calculated from duplicates within the experiments): RNA 1.72, DNA 0.051.

Per average cell	Stages of population growth (1)	(2)	(3)	(4)
Dry weight ($\mu g \times 10^{-3}$)	4.1	8.2	9.8	5.1
RNA ($\mu g \times 10^{-5}$)	24.6	53.3	68.4	33.9
DNA ($\mu g \times 10^{-5}$)	1.36	3.02	3.84	1.97
RNA (% of dry weight)	6.0	6.5	7.0	6.6
DNA (% of dry weight)	0.33	0.37	0.39	0.39
$\frac{\text{DNA}}{\text{RNA}} \times 100$	5.5	5.7	5.6	5.8

TABLE II

Molar ratios of the bases of RNA at different growth stages.

Nos. 1–4 refer to different growth stages: control (1), after heat treatment (2), prior to (3) and after (4) the first synchronous cell division. S.E. is the standard error as estimated from duplicates. v is the approximate coefficient of variation on a percentage basis. Two parallel experiments are represented.

m.r. = μM/100 mg dry weight	Stages of population growth									
	(1)		(2)		(3)		(4)		S.E.	v (%)
	m.r.	%	m.r.	%	m.r.	%	m.r.	%		
Adenine	5.77	30.9	6.3	29.8	6.60	30.4	6.39	30.8	0.104	1.68
Guanine	3.70	19.8	4.14	20.4	4.75	21.9	3.97	19.1	0.280	6.76
Cytosine	3.61	19.3	4.34	21.4	4.30	19.8	4.28	20.6	0.282	6.82
Uracil	5.61	30.0	5.75	28.4	6.05	27.9	6.12	29.5	0.360	6.12
Total	18.69	100.0	20.26	100.0	21.70	100.0	20.76	100.0	–	–

Dry weight and nucleic acids.—Table I shows the average values for dry weight, DNA and RNA as calculated in μg per average cell. Within each experiment the procedure of separation and estimation of the nucleic acids was carried out in duplicate, beginning after the acid hydrolysis of RNA and DNA. From the resulting duplicate values the standard error was calculated.

The dry weight doubles in the course of the treatment and increases further between the end of the treatment and the first division by about 25 per cent. Similar changes occur in the absolute amounts of RNA and DNA as calculated per average cell. This almost constant increase of the nucleic acids in the course of the treatment, when expressed on a percentage basis of the dry weight, is clearly seen from Table I. Surprising, however, is the relatively small DNA/RNA ratio, indicating that only about 5 per cent of the nucleic acid in the average cell is DNA.

Composition of the nucleic acids.—Table II shows the molar ratios of RNA as expressed in μM per 100 mg dry powder. From the normal control (No. 1) it appears that the ratios adenine/uracil and guanine/cytosine are almost unity. The total amounts of the former two compounds are about 50 per cent higher than the total amounts of the latter two compounds. The pattern of the composition of RNA seems to be the same in logarithmically growing cultures and in cultures exposed to the temperature treatment (No. 2), and during the physiological changes following the first synchronous cell division (Nos. 3 and 4).

The molar ratios of the DNA bases are presented in Table III. In the

TABLE III

Molar ratios of the bases of DNA at different growth stages.

Nos. 1–4 refer to different growth stages: control (1), after heat treatment (2), prior to (3) and after (4) the first synchronous cell division. S.E. is the standard error as estimated from duplicates. v is the approximate coefficient of variation on a percentage basis. Three parallel experiments were carried out.

m.r. = μM/100 mg dry weight	Stages of population growth								S.E.	v (%)
	(1)		(2)		(3)		(4)			
	m.r.	%	m.r.	%	m.r.	%	m.r.	%		
Adenine	0.394	36.6	0.423	35.3	0.426	33.5	0.463	36.9	0.0125	2.93
Guanine	0.123	11.5	0.139	11.6	0.161	12.6	0.130	10.4	0.0092	6.66
Cytosine	0.161	15.0	0.183	15.3	0.201	15.8	0.194	15.4	0.0072	3.90
Thymine	0.396	36.9	0.452	37.8	0.486	38.1	0.469	37.3	0.0078	1.73
Total	1.074	100.0	1.197	100.0	1.274	100.0	1.256	100.0	–	–

control (No. 1) the bases adenine and thymine are present in equimolar amounts and constitute about 70 per cent of the total DNA, while the guanine/cytosine ratio—contrary to the results in RNA—appears to be slightly in favour of cytosine. Just as with the molar ratios of the bases in RNA, the composition of DNA does not appear to be affected significantly by the temperature treatment. The purine/pyrimidine ratio keeps within the range of 1.00 to 1.10 for RNA and is between 0.86 to 0.93 for DNA.

DISCUSSION

It was shown that the DNA bases of the ascites tumor cells of the mouse could be separated in one dimension by paper chromatography [16]. Apparently due to UV-absorbing contamination of the DNA fraction, we were unable to separate the four bases in one dimension. In the second dimension, however, the unspecific absorption remained at the starting line, while the purine and pyrimidine bases moved according to their R_f values in this solvent. Contamination of the DNA fraction with RNA (about 5 per cent) as sometimes reported, could not be detected [16].

A predominance of RNA in the nucleic acid content of *Tetrahymena pyriformis*, strain H, has already been reported by Heinrich *et al.* [4] and Flavin and Engelman [3]. The figure for the DNA/RNA quotient given by these authors was found to be about 0.10. This value is, however, about twice as high as found by the method mentioned for the strain GL used in this investigation and may be due to different culture conditions [4, 14].

The DNA content of the average normal cell as reported here is in good agreement with the results obtained on an earlier occasion using a microbiological method [23]. However, in the synchronized cells the situation is somewhat different, in that we never obtained more than a slight doubling of the DNA content per average cell in the course of 6.5 hours of the heat treatment. This value is only about one half of that found when the cultures were exposed for 7.5 hours to the temperature treatment and assayed microbiologically [23].

The fact that the DNA content of a growing and multiplying cell is doubled in between two successive divisions [5] may lead to the assumption that DNA synthesis might be involved in the controlling mechanism of cell division. Careful theoretical analysis, based on such ideas, has already been presented by Rahn [11]. Another hypothesis in this direction was advanced by Lark and Maaløe for the explanation of synchronous cell division in *Salmonella typhimurium*, induced by temperature shifts [6]. These authors found that DNA syntesis, nuclear division and cell division are radically affected by temperature shifts, and arrived at the conclusion that cell division might be controlled by DNA synthesis [6]. However, studies by Falcone and Szybalski on the mechanism of synchronous cell division in *Bacillus megaterium* indicate that, in this system, DNA synthesis is least affected by suboptimum temperatures, as compared to the increase of other parameters of the cell, such as RNA, dry weight and nitrogen [2].

The analyses of the nucleic acids in *Tetrahymena* show no such effects of the intermittent temperature treatment on the concentration of DNA or RNA as expressed on a percentage basis of dry weight (acetone powder). Furthermore no change in the molar ratios of the bases of both nucleic acids in different growth stages could be observed. Significant shifts within the constituents of DNA were sometimes reported for tumor cells [16, 20].

Recently Ducoff investigated the effect of X-rays on normal and synchronized cultures of *Tetrahymena pyriformis*, strain W [1]. He arrived at the conclusion that the delay of cell division induced by irradiation might not be caused by inhibition of processes connected with DNA synthesis. We still have to keep the possibility in mind, at least where the effect of the temperature cycles is concerned, that the treatment might interfere with the formation or function of a very specific but quantitatively insignificant fraction of DNA, which would fall outside the limits of the method applied. Similar ideas were suggested by Prusoff *et al.*, resulting from studies of the effect of azathymidine on the synthesis of DNA [10].

SUMMARY

1. The bases of RNA and DNA were isolated from normal and synchronized cells of *Tetrahymena pyriformis* GL.

2. The dry weight (acetone powder) of the cells increases 100 per cent in the course of about 7 hours of treatment. It increases further 25 per cent within the hour between the end of the treatment and the first synchronous division.

3. The RNA is 6.0 per cent and DNA 0.33 per cent of the dry weight in normal cultures. These values are not changed significantly in the course of the treatment.

4. The molar ratios of the four bases of RNA and DNA appear to be constant throughout the treatment.

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THE INTRACELLULAR DISTRIBUTION OF MASS DURING ONTOGENY OF POLLEN IN *TRADESCANTIA* L.

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Observations on the distinctive form of mature pollen grains of many species of plants have contributed to such diverse disciplines as cytology, phylogeny, archaeology, petroleum geology, and immunology (allergy). The frequently complex form of the mature pollen grain makes it highly desirable that detailed information concerning ontogeny be available for critical interpretation of their structure and comparative morphology.

Routine cytological techniques have been applied in the analysis of pollen development in relatively few species [17, 35, 15, 16]. Various workers using standard methods (e.g. [29, 21]) have recorded the changing morphology of chromosomes, cytoplasm, vacuoles, etc. The general cytological pattern of pollen development in *Tradescantia*, as revealed by visual light microscopy, has been recorded by Sax and Edmonds [28], Taylor [33] *et al.* There is an increasing need, however, for more detailed and specialized analyses of various phases of the ontogenetic process particularly as they provide the basis for the interpretation of the ultrastructure and functions of the various components of the mature pollen grain.

The need for a complete analysis of the changes occurring during pollen ontogeny makes it desirable to apply a new physical technique for the determination of the dry weight (mass) of cellular structures. The technique here considered employs quantitative historadiography following the methods outlined by Engström [10] for the analysis of biological material at the cellular level. With suitable material Engström's method permits analysis of distribution of mass within a cell. The present communication provides a generalized description of the most striking features of pollen development in *Tradescantia* as revealed by historadiography.

The theory and principles of X-ray microradiography have been presented in detail by Engström [10] and Engström and Lindström [13]. The method utilizes the principle that in the region of soft X-rays (6 to 12 Å) the absorp-

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tion of the X-radiation is proportional to the mass per unit area of the biological sample, particularly with respect to the common mass of the elements carbon, nitrogen, and oxygen occurring in the specimen. Substantial variations in the proportion of these chemical elements have little effect on the final result. Similarly, such elements as Ca, Fe, K, P, S, and Mg may occur in concentrations significantly in excess of their usual amounts in biological materials without influencing determinations of mass (cf. [13, 12]).

The degree of accuracy of the method is dependent upon a number of factors, both physical and biological. In this connection the papers of Engström [10], Glick *et al.* [20], Lindström [22], Davies *et al.* [8], and Brattgård *et al.* [5] should be consulted. Since the ultimate aim of cytochemistry is the acquisition of reliable information concerning the localization, distribution, and nature of substances making up cells in relation to their biology, it is of utmost importance, wherever possible, to follow procedures which minimize the errors resident both in the technique and in the prepared biological specimen itself (cf. [18]).

With respect to the X-ray technique, various factors contributing to experimental error have been described in detail by Lindström [22]. The accuracy of the determination of the mass of the reference system, for example, is a factor of fundamental importance (cf. [4, 3]). In the present study, reference foils were prepared only from areas of the nitrocellulose film selected for uniformity of interference characteristics. In such regions, areas 1 centimeter square were cut out and weighed. These weighed areas were then cut into narrow strips and used in preparing the reference foils.

Ideally the specimen, e.g. the cell, should be living. Since this is frequently not possible with many procedures, great care must be exercised in order that the variously preserved cells may exhibit as few artefacts as possible. Further the artefacts should be capable of interpretation with reference to the methods employed. Reagents or operations which contribute to extraction or migration of cellular components should be held to a minimum in any project directed toward a definition of the intact cellular system.

An obvious source of error in the present study stems from the nature of the material which necessitated the use of fixation, embedding, and sectioning. The reagents employed have undoubtedly introduced artefacts in the cells used for X-ray analysis. However, even with such limitations, observations can be of interest particularly when viewed with reference to data obtained from researches employing different techniques. Throughout the present investigation the morphology of the fixed cell was compared with living cells and cells fixed by different techniques.

MATERIALS AND METHODS

A clone (line 6-50) of *Tradescantia paludosa* E. Anders and R. E. Woodson grown with accessory illumination in the greenhouses of the Cell Research Institute, Stockholm, was used in this study. This clone exhibits the usual six bivalent chromosomes and is free of visible fragments of chromatin during meiosis. Meiocytes of various stages of development from prophase I (pachytene) through telophase II were prepared for analysis by placing anthers of known stages into Carnoy's reagent modified as follows: propionic acid 25 per cent; chloroform 30 per cent; absolute ethanol 45 per cent. The fixation period was 20 hours. Evacuation was used to facilitate penetration of the fixative. The meiocytes preserved in this manner were brought into 56–58°C melting-point paraffin via chloroform solutions, up to 15 per cent paraffin at room temperature, followed by a minimal period (usually ca. 8–10 hours) in the paraffin oven. The most desirable section thickness was found to be in the 4 to 6 μ range.

It is of interest that our attempts to obtain satisfactory preservation of pollen-mother-cell structure in 5 per cent formalin and in isopentane at —160°C (for freezing-drying procedure) were not successful. Improvements in various methods for freezing-drying could well be applied in the future for trial with such materials [2, 19].

Selected sections of pollen-mother-cells in paraffin were permitted to spread for a brief period on the surface of a distilled water bath at ca. 48°C. The circular brass holder, coated with the thin specimen-supporting film of collodion, was carefully submerged below the floating, expanded paraffin sections. The specimen-holder was brought to the surface in such a manner that the expanded sections were oriented on the film over the slit-aperture (Fig. 20). After drying, usually at least 2 or 3 hours at room temperature and humidity, the paraffin was removed with chloroform. The reference system of thin films or foils of known mass was then placed beside the specimen. The arrangement of the thin reference films alongside the biological specimen can be a matter of some difficulty since static electricity, dust-particles, and subsequent folding can be involved. In Fig. 22 a fold or ripple in the reference system is shown. The microradiograms were then made, with an exposure of ca. 14 minutes at 60–130 m amp. and 2.25 k volts using Lippmann film (75260-2LD and 75260-2NE) supplied by Gevaert, Antwerp. Paraffin sections on either side of the sections utilized for X-ray microradiography were retained for usual staining and visual light microscopy. In some instances, sections were also saved after irradiation for transfer to slides for Feulgen staining allowing the use of identical cells in photometry and micrometry. The difficulty involved in the transfer, orientation, and staining of these deparaffinized tissues precluded success in some cases. The exposed film was developed for 15 seconds at ca 20°C in the developer used by Engström and Lindström [13]. In an effort to minimize grain size, this combination of long exposure and short development was used. In order to insure that the irradiation over the photographic field was of uniform intensity, blanks of photographic film were run

periodically. The finished microradiograms were mounted on slides, usually with a surrounding opaque mask, for microscopical analysis. Favorable cells were selected for the photomicrographs included in this account.

RESULTS

In the case of the material here described, the earliest developmental stage studied was the pachytene stage of prophase I. The earlier leptotene stage during which the chromosomes are very slender and much entangled has not been analyzed as yet.

At the pachytene stage of meiosis, the fairly long, irregularly oriented, bivalent chromosomes showed an absorption which was greater than that of the remainder of the cell (Fig. 2, Table I). A Feulgen preparation of a similar pachytene locule is shown in Fig. 1. At this stage the bivalent chromosome is ca. 0.8–1.3 μ in diameter. This dimension indicates in effect the level of resolution. With the X-ray abosrption technique, the character of the granu-

TABLE I

Ontogeny of *Tradescantia paludosa* Pollen $\left(\text{mass per unit area } \left[\frac{\times 10^{-11} \text{ grams}}{\text{sq. microns}}\right]\right)$.[1]

Cell components	Microsporocyte meiosis				Microspores		Mature pollen grains
	Pachytene	Diakinesis	Telophase I	Telophase II	Uninucleate	Mitosis	
Cell wall	0.70	2.11	4.63	9.25	2.51	2.74	5.83
Cytoplasm	0.70	2.11	4.63	4.22	2.51	2.74	5.83
Vacuole	–	–	–	–	0.44	–	–
Karyolymph	0.28	0.09	–	–	–	–	–
Nuclei	–	–	4.63	4.22	2.51	–	2.96
Chromosomes	1.98	4.82	–	–	–	1.79	
Nucleolus	–	–	–	–	2.51	–	–
Tapetum	–	2.12	2.32	2.46	–	–	2.09

[1] The values reported in Table I were derived from equation (10a) provided by Engström and Lindström [13] and Lindström [22]. The basis for expressing our data in terms of mass per unit area was established as follows. Carefully selected cells were drawn on precision graph paper with the aid of camera lucida. The weight per sq.mm of each sheet of graph paper was determined experimentally. Desired portions of the drawings were cut out and weighed. These weights were converted to area and then used in equation (10a) in the calculation of mass for the cellular components involved.

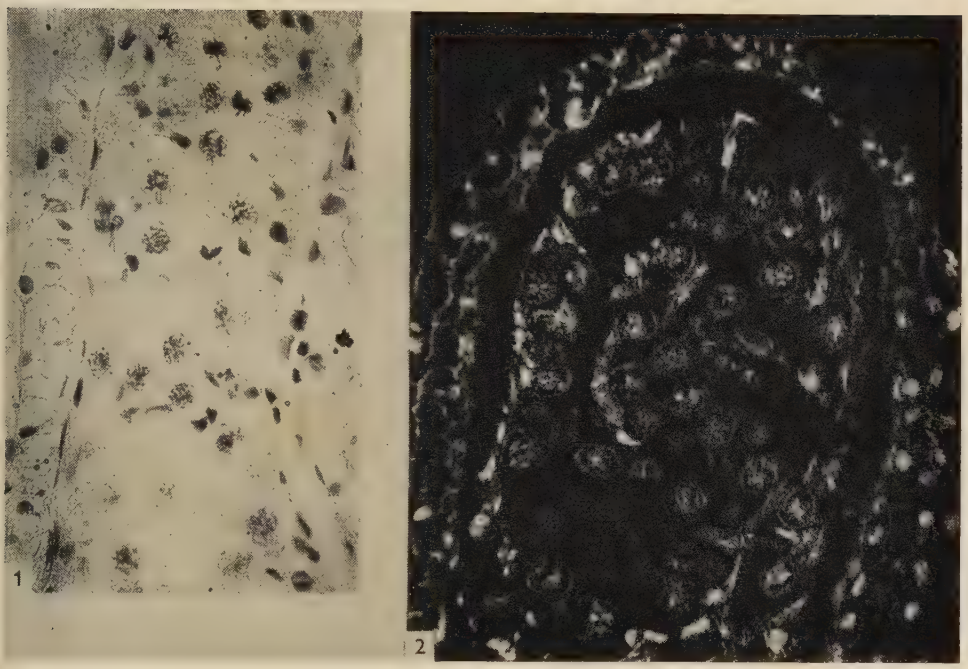


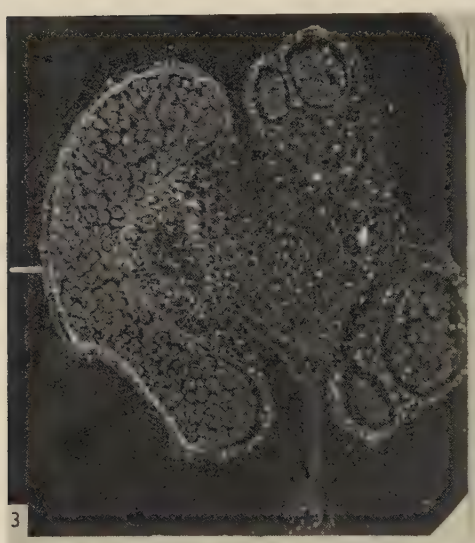
Fig. 1. Prophase I (pachytene); Feulgen stain. × 300.
Fig. 2. Prophase I (pachytene); microradiogram of same stage as Fig. 1 in a similar but not identical location. × ca. 275.

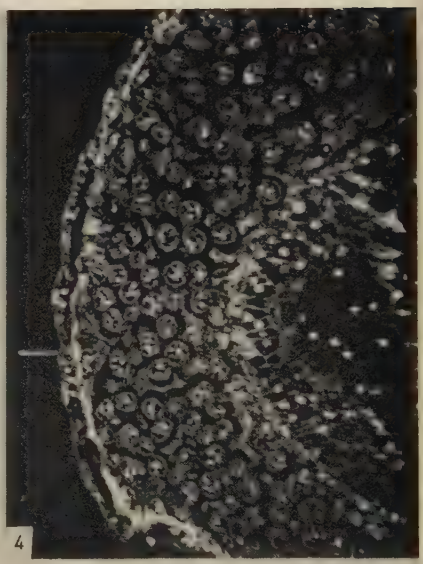
larity of the film upon which the X-ray image of the cells is initially recorded, at a 1 : 1 ratio, is of fundamental importance in establishing the limit of resolution of cytological structure.

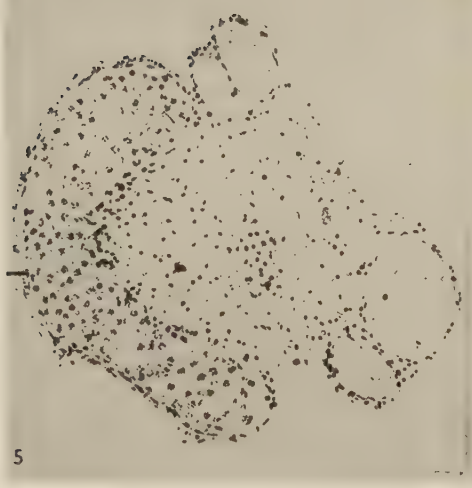
During subsequent development in prophase I the absorption by the chromosomes increases markedly until at diplotene-diakinesis the much-shortened chromosomes are the most X-ray absorbing structures within the cell (see Figs. 3, 4, 7, 8, 9, Table I).

The distinctive opacity of the chromosomes to X-rays could be followed until the later phases of meiosis. Beginning with late anaphase I–early telophase I, the extranuclear portions of the cell show increasing X-ray absorption, apparently attaining almost the same opacity characterizing the prophase chromosomes. Thus the X-ray image, at such periods, reveals a cell appearing rather uniformly absorbing and structureless since the individual components are no longer differentiated by X-ray absorption characteristics (see Figs. 10, 12, Table I).

During cytokinesis, at telophase I and II, the newly formed cell walls are







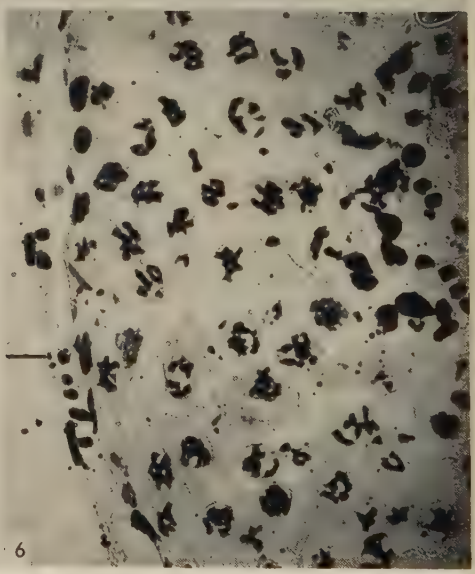


Fig. 3. Prophase I (diplotene-diakinesis); microradiogram of complete anther section. × 118.
Fig. 4. Portion of radiogram shown in Fig. 3 at enlargement of × 155. White line refers to mound of tapetal tissue which may be used as reference point for locating identical cells in Figs. 3 through 7.
Fig. 5. Feulgen-stained preparation of section shown in Fig. 3. This section was transferred from the X-ray specimen holder to a microscope slide. Pollen-mother-cells in the locules to the right were lost during this transfer. × 155.
Fig. 6. Enlarged portion of left locule of Fig. 5 in marked region. × 300.

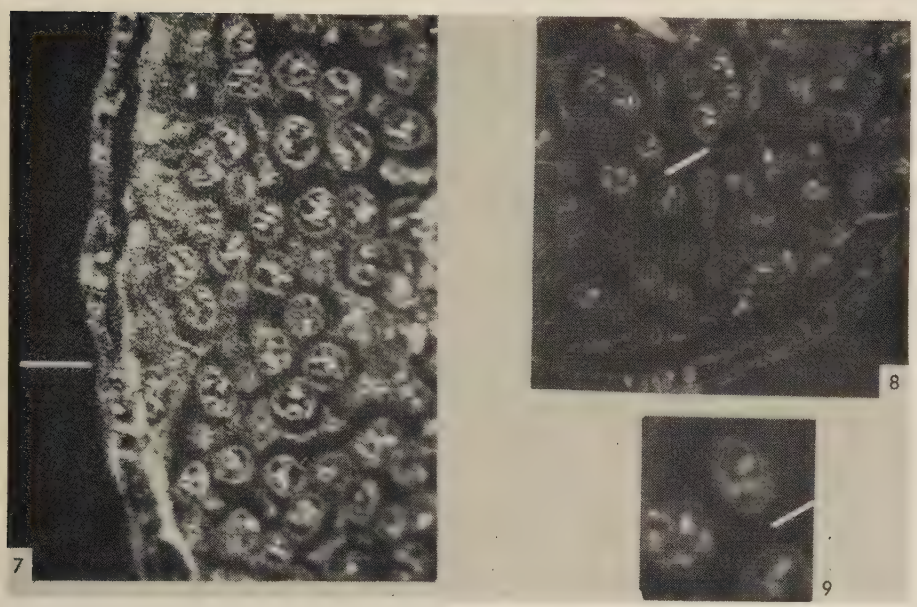
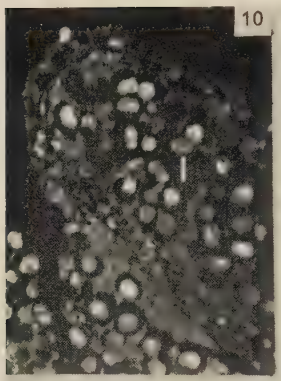
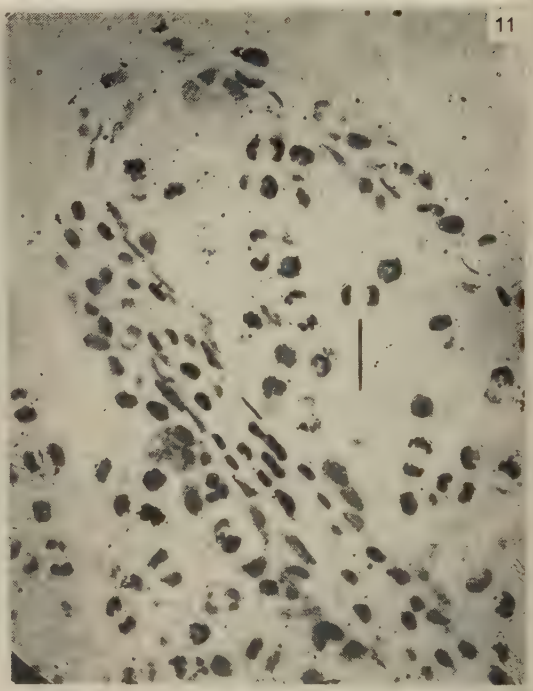


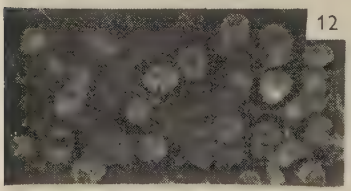
Fig. 7. Guide line indicates location of cells identical with those shown in the Feulgen preparation (Fig. 6). Microradiogram; × 290.
Fig. 8. Prophase I (late diakinesis); × ca. 250. Note separated, compact chromosomes showing high absorption.
Fig. 9. Three cells indicated by marker in Fig. 8 at magnification of × ca. 480. Note that the chromosomes are individually resolved despite the great enlargement.

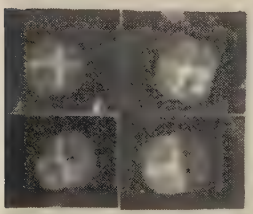
sharply differentiated from adjacent regions by heavier absorption characteristics (Figs. 12, 13, Table I). This seems especially notable during the quartet or tetrad stage at telophase II (Figs. 13, 14, Table I).

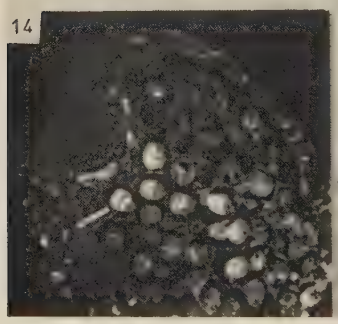
At the completion of telophase II, each initially diploid pollen-mother-cell has become resolved into the quartet of four haploid microspores. At the end of telophase II with the development of the microspore, a distinctive vacuome is organized (Figs. 16, 17). As expected, this vacuome shows minimal X-ray absorption. Within the nucleus of the microspore, the nucleolus begins to be differentiated by its absorption characteristics. In some cells during early prophase of microspore mitosis, the nucleolus attains heavy absorption (Fig. 23). The chromosomes share the heavily-absorbing X-ray characteristics of the nucleolus during the early aspects of microspore mitosis. The data in Table I indicate that the univalent chromosomes of microspore mitosis approach the absorption values recorded for pachytene bivalents. Figs. 24 and 25 provide a comparison of a microradiogram and a Feulgen preparation of identical microspores during mitosis. The nucleolus as











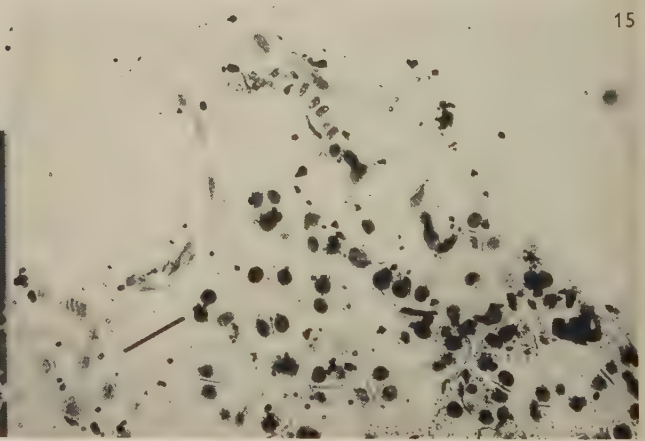
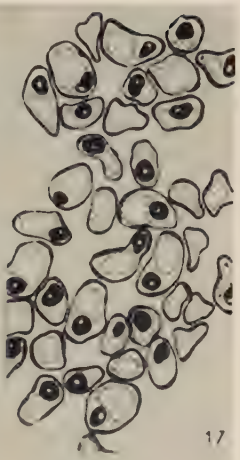
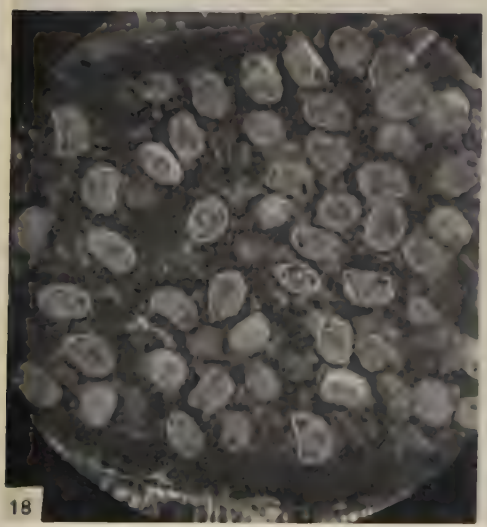


Fig. 10. Telophase I; microradiogram. Guide line refers to pollen-mother-cell showing high X-ray absorption of cell-plate. × 155.
Fig. 11. The identical cells shown in Fig. 10 after transfer to microscope slide and Feulgen reagent. × 300.
Fig. 12. Telophase I; microradiogram. Note sharply absorbing cell-plate areas. × ca. 225.
Fig. 13. Telophase II; microradiogram. Note the heavily absorbing cell-walls separating the individual components (microspores) of the quartets (tetrads). × ca. 435.
Fig. 14. Telophase II; microradiogram. × ca. 155.
Fig. 15. Only specimen of Telophase II (section shown in Fig. 14) surviving transfer to Feulgen reaction. Guide lines indicate identical cells. × 300.





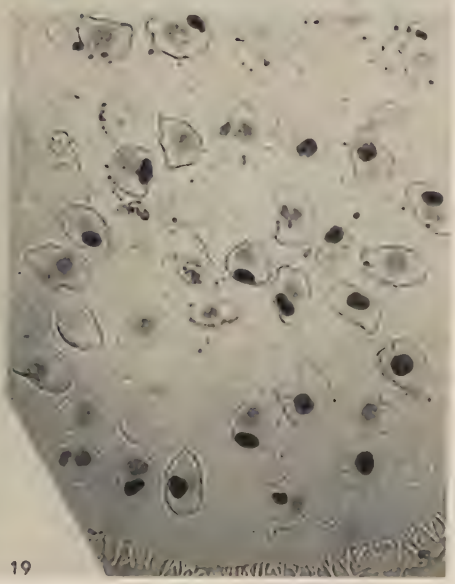
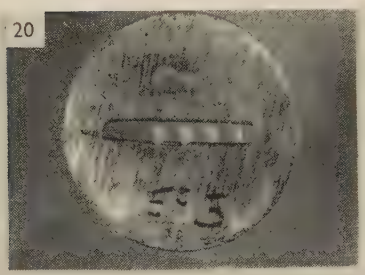


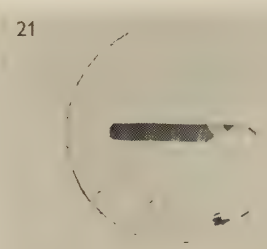
Fig. 16. Telophase II; separated microspores; microradiogram. The break in the wall of the locule to the right represents the incision made to facilitate rapid penetration of fixative. Note the heavy X-ray absorption of the thickened areas of cell walls of the anther wall. The developing vacuome is recorded by low X-ray absorption. This stage is poorly preserved by the fixative. × ca. 155.

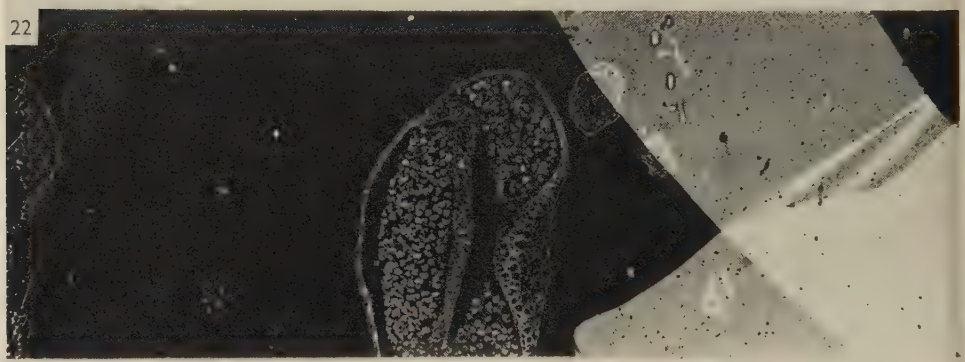
Fig. 17. Tracing of microspores from photomicrograph (using Manton technique) of Feulgen preparation of cells from adjacent section of same locule (to right) as Fig. 16. × 300.

Fig. 18. Young, binucleate pollen grain; microradiogram. Note: the intense absorption of nucleoli of tube nucleus; low absorption of extranucleolar regions of nuclei; comparatively high absorption of cytoplasm; low absorption in vacuoles. × ca. 230.

Fig. 19. Feulgen preparation of cells in adjacent section of same locule as Fig. 18. Note light-staining tube nuclei with conspicuous nucleoli; dark staining immature generative nuclei. × 300.







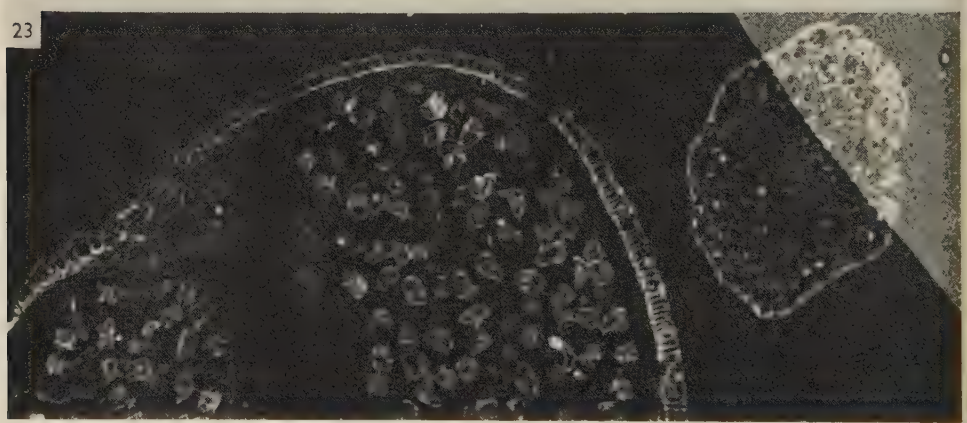


Fig. 20. Brass specimen holder with four sections of anther arranged on supporting film in slit aperture. × 2.

Fig. 21. Microradiogram recorded on punched out disk of Lipmann film (emulsion No. 75260-2LD) prepared from the completely assembled specimen-holder shown in Fig. 20. The X-ray image corresponds to the specimen holder exactly as opposing pages of a book. The reference system which is located in the slit aperture to the left of the sections in Fig. 20 is plainly evident in the X-ray image. × 2.

Fig. 22. Photomicrograph of the microradiogram shown in Fig. 21 illustrating portion of reference foils and adjacent anther section. × 37.

Fig. 23. Photomicrograph of microradiogram shown in Fig. 21. × 155. Note the individual microspores, which are entering mitosis, and show minimal absorption in the vacuole. The X-ray absorption of a single thickness of the reference foil is included in the upper right corner.

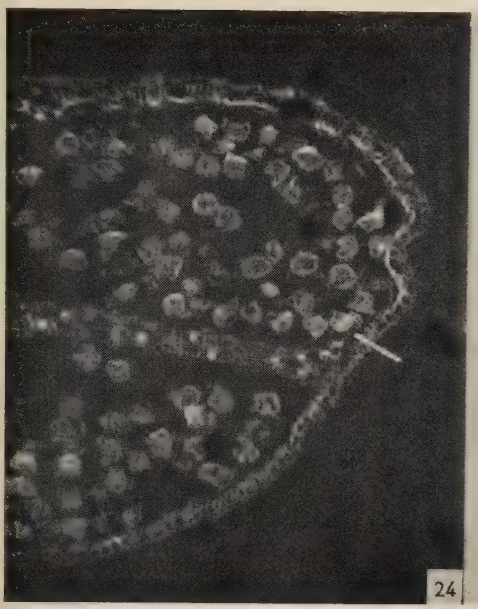
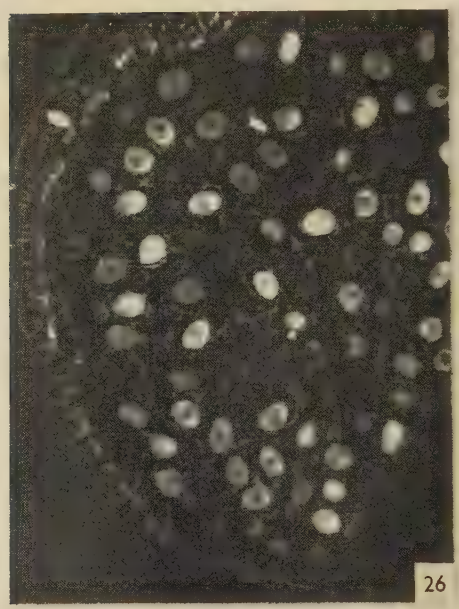
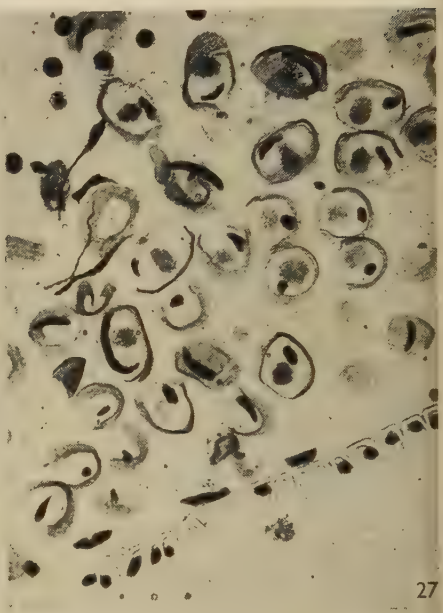


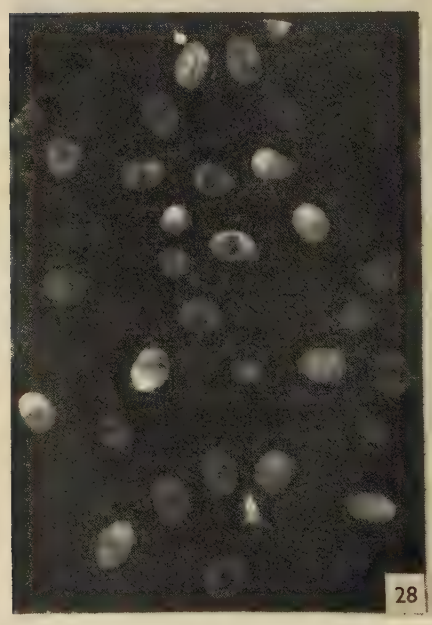
Fig. 24. Microspores, various stages of mitosis. Microradiogram. Line refers to anaphase stage also shown in Fig. 25. Within the limits of resolution, this microspore shows high absorption in the chromosomes and adjacent regions. × 155.
Fig. 25. Feulgen preparation of identical section shown in Fig. 24 after production of the X-ray microradiogram. Some cells in the upper portion of the locule were lost in transferring specimens from holder. × 300.

a structure vividly differentiated by heavy X-ray absorption is also seen in the ensuing binucleate stage. In this early, binucleate pollen grain, the nucleolus of the tube nucleus stands out sharply as the only nuclear structure having relatively high absorption characteristics. These features are clearly evident in the microradiogram illustrated in Fig. 18. This figure if considered alone would suggest that the cells were *uninucleate*. However, reference to Fig. 19, which illustrates stained cells from the same locule, documents the stage as that of the young *binucleate* pollen grain. The relatively low absorption of the immature generative nucleus and the extra-nucleolar regions of the tube nucleus is highly noteworthy.

As subsequent development proceeds, X-ray absorption of the nuclear components, including the nucleoli, becomes relatively reduced (cf. Fig. 26, Table I). This trend continues until the appearance of the fully mature pollen grain with two morphologically differentiated nuclei. The common circumstance in the microradiograms is one showing relatively high absorption in







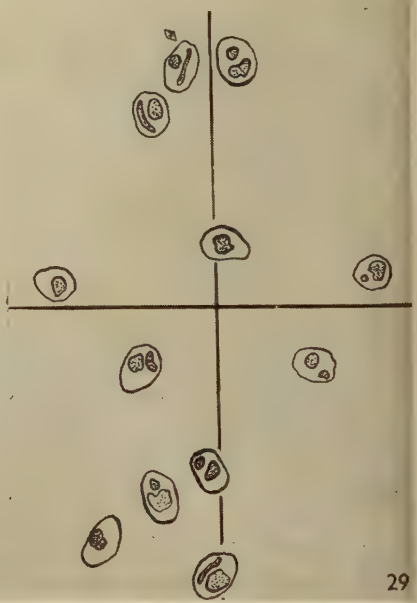


Fig. 26. Microradiogram of mature, binucleate pollen grains. High X-ray absorption is evident in extranuclear regions. Nuclei are seen as dark (low-absorbing) areas. × 160.
Fig. 27. Feulgen-stained preparation of similar, but not identical, pollen grains of the same section used for Fig. 26. In some pollen grains the elongate intensely-stained generative nuclei and the spheroidal lighter-stained tube nuclei are both visible. × 315.
Fig. 28. Microradiogram illustrating low X-ray absorption of generative and tube nuclei of mature pollen grains. × 240. (Cf. Fig. 29.)
Fig. 29. Individual pollen grains of Fig. 28 showing nuclear areas (regions of low absorption) in outline. × 240.

the cytoplasm with comparatively low absorption in the two nuclei (Figs. 27, 28, and 29, Table I). It is at this point that anthesis takes place. The mature pollen grains after being released from the anthers are capable of rapid germination under suitable conditions.

DISCUSSION

The technique used in the present study is so specialized and relatively untried that there is especial need for correlation with more orthodox techniques. Previous studies [28, 29, 33, 21] reveal a series of changes in cell shape and volume as well as in the morphology of nuclei and the vacuome at various stages of pollen development. During the final phases of pollen maturation, changes occur chiefly in extranuclear regions, including metabolic granules, and the cell wall. At maturity the pollen grain wall attains great complexity as is evidenced from studies of thin sections and surface replicas [1, 7, 24].

In terms of the total cellular complex the observations on variation in intracellular mass will need to be correlated with other cytophysical and cytochemical investigations. Studies have been made of nucleic acids in pollen-mother-cells by Painter [26], Taylor and McMaster [34], Swift [31, 32], Bryan [6], Plaut [27], and others. These reports indicate that synthesis of desoxyribose nucleic acid takes place during the interphase stages occurring before leptotene in the microsporocyte, in the microspore, and in the pollen grain. In our studies the last two of these interphase stages exhibit increasing X-ray absorption in the cytoplasm.

General chemical analyses based on air-dry samples of mature pollen of relatively few species have been made [23]. Nielsen *et al.* [25] observed sulphate ash, carbohydrates, lipoids, phosphorus, amino acids, and vitamins in pollen of four species. Such chemical analyses are naturally without reference to specific intracellular association. It follows that attempts to interpret the present data on distribution of mass in *Tradescantia* pollen-mother-cells in terms of chemistry would be premature.

In our analysis the most evident trends with respect to absorption of soft X-rays, and therefore with respect to overall structural mass (cf. [10, 11, 14]) have been recorded during meiosis in pollen-mother-cells and subsequent microspore mitosis. The comparative relationships of mass (X-ray absorption) of nuclear and extranuclear structure of the individual cell undergo reversal during ontogeny. Early in ontogeny, greatest relative mass is recorded in the chromosomes. Later, the nucleolus is observed to be prominently

absorbing, whereas the extranucleolar portions of the nucleus are not. Finally in the mature pollen grain it is in the cytoplasm and wall of the cell that the highest absorption occurs.

It is of interest to note that according to Sparrow [30] the periods of greatest sensitivity of protoplasm to X-rays in terms of chromosome breakage in living cells of *Trillium* occur during stages characterized by condensed chromosomes. Our X-ray absorption data cannot be clearly associated with such observations. There is the suggestion, however, that stages of high sensitivity to chromosome breakage, pachytene through metaphase I, are also those during which greatest cellular mass is localized in the chromosomes.

It should be noted that the present observations apply to material fixed in modified Carnoy's reagent and are subject to the limitations which this solution imposes. The deficiencies of Carnoy-type fixation were particularly evident in the uninucleate microspore of our material (Fig. 16).

Davies *et al.* [9] by means of interference microscopy have studied the development of living microspores into pollen grains in *Tradescantia bracteata*. It seems especially significant to note that they found in their material that during interphase the nucleolus includes an outer region of higher dry weight per unit volume than any other portion of the nucleus. Their report of similar optical retardation for the two vacuoles in the microspore just prior to nuclear division agrees with the X-ray absorbing characteristics of these structures (see Fig. 23). Further, in their study, cytokinesis was observed in staminal hair cells in which the cell-plate and newly developing cell wall were evident as a line of high mass per unit area. In our material this circumstance is recorded in the distinct and localized X-ray absorption seen in the cytokinetic phases of the microsporocytes (Figs. 12, 13).

Many phases of the developmental picture are still incompletely analyzed, e.g. changes in the tapetum and in the organization of the pollen wall. A comparative study of the tapetum, which is so closely associated physically with pollen-mother-cells during ontogeny, is contemplated for the future.

SUMMARY

1. Changes in the distribution of intracellular mass during ontogeny of pollen grains, as recorded by the application of Engström's X-ray absorption technique, are reported for the first time.

2. During development of the mature pollen grain the greatest relative mass is associated with the chromosomes early in the process (pachytene through metaphase I).

3. The extranuclear portions of the cells attain absorption (mass) characteristics similar to those of early prophase I chromosomes beginning approximately at late anaphase I–early telophase I.

4. Cytokinesis, at telophase I and II, is strikingly associated with sharply localized heavy X-ray absorption in the newly-formed cell walls.

5. By the end of telophase II, each microsporocyte has produced four microspores whose distinctive vacuome is recorded by minimal X-ray absorption.

6. In the microspore, and particularly in the vegetative (tube) nucleus of the early binucleate pollen grain, the nucleolus becomes distinctly differentiated from the remainder of the nuclear structures by its comparatively intense X-ray absorption.

7. During the later phases of ontogeny of the pollen grain, there appears to be a gradual increase in mass (X-ray absorption) in the cytoplasm.

8. In the mature binucleate pollen grain high X-ray absorption is associated with extranuclear structure (cytoplasm and cell wall). Minimal absorption is recorded at this stage in both of the nuclei.

9. Specific difficulties encountered in investigating microsporocyte (pollen-mother-cell) material with this X-ray technique are briefly reviewed.

The basic phases of this research were completed in Professor Arne Engström's laboratory. His never-failing encouragement has been of inestimable value. Professor T. Caspersson extended many courtesies of the Institute for Cell Research throughout the senior author's residence in Stockholm while on sabbatical leave from the University of Minnesota. Dr. Bo Lindström gave helpful advice. Professor David Glick generously made photometric apparatus available. He has also provided invaluable aid by critically reviewing the manuscript. To all these persons, our sincere appreciation is warmly extended.

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REACTIONS OF C^{14}-LABELED CARCINOGENIC AZO DYES WITH RAT LIVER PROTEINS

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When the carcinogenic azo dye 3′-methyl-4-dimethylaminoazobenzene is administered to rats in short-time experiments, the dye becomes bound at a particularly rapid rate to the proteins of the submicroscopic structures of the liver cells [14, 15]. It is well known that these structures play a fundamental part in the protein metabolism of the liver [13, 7, 26, 17]. It is therefere suggestive to imagine that the dye by reacting with the proteins of these structures may interfere with mechanisms responsible for the controlled formation of the cell-specific proteins.

The binding of azo dyes to protein is probably a result of metabolic transformations of the dye. It has been shown by the Miller group [22] that the carcinogenic azo compounds are metabolized by rat liver in three different ways: (1) by reductive cleavage of the azo bond, (2) by hydroxylation, and (3) by demethylation, the last methyl group being detached from the amino group by oxidation.

The reductive cleavage of the azo bond has been studied *in vitro* in a particulate liver system containing microsomes, with reduced triphosphopyridine nucleotide (TPNH) as the reducing agent [23]. The oxidative degradation has also been studied *in vitro* [24] in whole rat liver homogenates supplied with hexose-1,6-diphosphate, diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). As far as we are aware, pure microsomal systems have not been tried in the oxidation reactions. The very interesting experiments by Axelrod *et al.* [2, 8] on the detoxicating systems of rat liver microsomes suggest, however, that microsomal elements and TPNH may be involved also in these transformations.

The object of this investigation was to further evaluate the role played by the microsomal material in the formation of azo-proteins. The initial binding to proteins of carcinogenic azo dyes was studied *in vivo* and *in vitro* in isotope experiments of very short duration. It was observed that C^{14} from isotopic azo dyes (labeled in the aniline ring) was rapidly bound to the proteins of liver homogenates, and also to isolated microsomes, in the presence of TPNH.

EXPERIMENTAL

Male albino rats weighing 175–200 g were used in the experiments *in vivo*, while rats weighing about 250 g were used for the preparation of liver homogenates. In a number of experiments the rats had been maintained for 2–8 weeks on a low protein diet [12] containing 0.06 per cent 4-dimethylaminoazobenzene (DAB). In other experiments the animals had been maintained on a low protein diet without DAB, or on a standard diet. Before the experiments the rats were kept without food for 15–20 hours. Albino mice were used in a few preliminary experiments *in vitro*.

C^{14}-labeled DAB and 4-monomethylaminoazobenzene (MAB) were synthesized from C^{14}-aniline (0.5 mc/m*M*), and were purified by chromatography on alumina columns [21]. C^{14}-aminoazobenzene (AB) was prepared from the labeled MAB by oxidative demethylation *in vitro* and subsequent repeated chromatographic purification in the presence of unlabeled MAB. The corresponding unlabeled compounds were also synthesized and purified by chromatography. Fructose-1,6-diphosphate, glucose-6-phosphate, isocitrate, TPN, TPNH, DPN, DPNH, and glucose-6-phosphate dehydrogenase were furnished by Sigma Chemical Company.

In the experiments *in vivo*, 2.5 mg of the labeled dyes were suspended in 1 ml of 0.9 per cent NaCl by means of a small amount of the detergent Tween 80. The suspensions were introduced into the rats by intravenous injections into the tail. The rats were killed by decapitation 3 minutes to 5 hours after the injections of the labeled, carcinogenic agents. The livers were perfused *in situ* with ice-cold 0.25 *M* sucrose, containing 0.005 *M* versene. During the perfusion the livers were chilled with ice. The perfused livers were minced and homogenized in the sucrose–versene medium, and cytoplasmic fractions were prepared by differential centrifugation, as has been described previously [14].

For the preparation of the protein samples the cytoplasmic fractions were treated by a modified Schneider procedure [25]. The dry protein samples were suspended in ether–chloroform, and were allowed to settle on aluminum plates (1 cm^2). The activity values observed were recalculated for indefinite sample thickness on the basis of an empirical activity saturation curve.

In the experiments *in vitro* the livers were carefully minced and washed in ice-cold 0.155 *M* KCl. They were then homogenized in a chilled glass homogenizer with 2.5 volumes of the same medium. Cell-fragments and nuclei were removed from the homogenates by centrifugation at 800 g for 10 minutes. The homogenates were in most experiments freed from mitochondria by centrifugation at 10,000 *g* for 8 minutes. When the mitochondria should be used in the experiments, the mitochondrial sediments were washed by centrifugation in 0.25 *M* sucrose. Microsomal and cell fluid fractions were separated by centrifuging the mitochondria-free homogenates for 50 minutes at 105,000 *g*. The microsomal fractions were washed by centrifugation.

The oxidative demethylation of MAB was studied under varying conditions. In standard experiments the incubation mixture contained, in a final volume of 2 ml, 10 μM $MgCl_2$, 50 μM potassium phosphate pH 7.6, 120 μM nicotinamide, 1–2 μM TPNH, 0.25–0.5 μM MAB (added in 0.1 ml of ethanol), and 0.4–1.0 ml of a 10 per cent liver homogenate in 0.155 *M* KCl, or equivalent amounts of isolated cytoplasmic fractions. The incubation samples were shaken in open test tubes at 37°C. After the

incubations the reaction was stopped by the addition of acetone. The dyes were extracted with benzene and analyzed by chromatography on alumina columns by the method of Mueller and Miller [24].

The binding of MAB-C^{14} to the proteins of liver cell constituents was studied under similar conditions as the demethylation reaction. Of the labeled dye 0.25 μM, dissolved in 0.05 ml of 95 per cent ethanol, was added to the medium of each incubation sample. In standard experiments 0.8 ml of a 30 per cent liver homogenate (or equivalent amounts of isolated cytoplasmic fractions) were added to the samples immediately after the azo dye. The reaction was stopped by the addition of acetone (3 volumes), and the samples were thoroughly shaken with acetone–benzene in a shaking-machine. The benzene layer was entirely removed after centrifugation, and one volume of ethanol was added to the water phase. The precipitated proteins were extracted in a Sohxlet extractor for 17–20 hours with methanol, and afterwards with ether. Zero time controls were included in all experiments. The specific activity of these controls were constantly well below 5 c.p.m. Since extraction of the samples with hot trichloroacetic acid effected only a minor reduction of the specific activities, such extractions were generally omitted in these series of experiments. The proteins were finally plated and counted in the same way as in the experiments *in vivo*. In several cases the metabolic degradation of the labeled dye was analyzed in the same incubation samples by chromatography.

RESULTS

By the use of the labeled azo compounds it was possible to study *in vivo* the intracellular distribution of the protein-bound isotope within a few minutes after the intravenous injection of the dyes (Table I). Irrespective of the individual variation between single experiments a particularly high isotope content was constantly observed in the proteins of the microsomes The activity maximum was reached in all fractions as soon as 6–30 minutes after the injections. In order to study the incorporation process during the course of actual tumor induction most experiments were performed on animals which had been maintained for 2–4 weeks on a low protein diet containing 0.06 per cent DAB. A few experiments were performed on rats which had not previously been treated with azo compounds. Slightly lower incorporation was generally observed in such animals.

As an introduction to the experiments *in vitro* on the binding to proteins of isotope from labeled azo dyes, the oxidative demethylation of MAB was studied in homogenates and in isolated cytoplasmic fractions of liver. In agreement with the previous observations of Mueller and Miller [24] MAB was rapidly metabolized by liver homogenates which had been fortified with fructose-1,6-diphosphate, DPN, TPN, and nicotinamide. The metabolic degradation proceeded at a rate proportional to the concentration of the

Table I

Incorporation *in vivo* of C^{14}-labeled carcinogenic azo dyes into the proteins of rat liver fractions. Cytoplasmic fractions prepared in sucrose–versene. C.p.m. per cm^2.

Diet of rats	Azo dye injected	Duration of isotope treatment	Mitochondria	Microsomes		Cytoplasmic fluid	
				Heavy	Small	Ca-insoluble fraction	Ca-soluble fraction
Low	C^{14}-DAB	3 min.	–	13	21	5	10
protein	C^{14}-DAB	5 min.	36	60	104	11	–
+ DAB	C^{14}-MAB	5 min.	41	61	128	34	36
	C^{14}-DAB	6 min.	76	110	190	37	46
	C^{14}-DAB	10 min.	66	92	141	42	60
	C^{14}-DAB	20 min.	57	100	168	56	116
	C^{14}-DAB	30 min.	46	61	86	46	73
	C^{14}-DAB	1 hr.	41	134	124	37	53
	C^{14}-DAB	4 hr.	43	83	81	41	42
Low	C^{14}-DAB	20 min.	38	50	71	14	16
protein	C^{14}-DAB	1 hr.	10	36	–	20	22

homogenate (Figs. 1, 2) for at least 45 minutes at 37°C. By chromatography on alumina [24] about 50 per cent of the metabolized dye was recovered as AB.

When TPNH was included in the reaction suspension instead of TPN and fructose-1,6-diphosphate, the cell fluid fraction was no longer required in the reaction. Under these conditions it was observed that the enzymatic system responsible for the oxidative demethylation was mainly localized in the microsome fraction (Table II). The considerably lower activity observed in the mitochondrial fraction may be partially due to contaminations of microsomal origin. DPNH could be utilized by the system, but to a lesser extent than TPNH (Fig. 3). The optimal pH-range for the demethylation of MAB by microsomes and TPNH was 7.5–7.9 (Fig. 4).

Microsomes from rats which had been maintained on DAB diet for 8 weeks showed, in the cases investigated, a somewhat higher capacity for MAB-demethylation than microsomes from standard rats.Mouse liver homogenates and mouse liver microsomes had almost as high capacity for demethylating MAB as the corresponding rat liver preparations (Fig. 2, Table II).

Sulfhydryl groups may be involved in the demethylation reaction, since a strong inhibition was observed in the presence of dihydroxidinaphthyldisulfide (10^{-3} *M*).

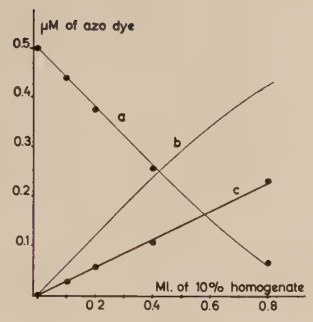

Fig. 1.

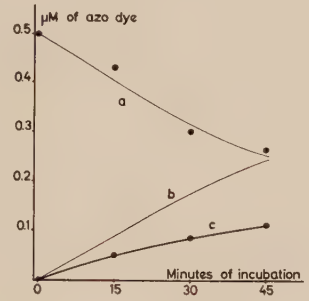

Fig. 2.

Fig. 1. Metabolic degradation of 4-monomethylaminoazobenzene (MAB) by a cytoplasmic homogenate of rat liver. Amounts of (*a*) MAB, (*b*) total metabolized MAB, and (*c*) 4-aminoazobenzene (AB) after incubation. The incubation system contained in final volumes of 2.0 ml: 12 μM fructose-1,6-diphosphate, 0.2 μM DPN, 0.2 μM TPN, 120 μM nicotinamide, 10 μM $MgCl_2$, 50 μM potassium phosphate pH 7.6, 100 μM KCl, and 0.5 μM MAB. Incubation time (37°C) 50 minutes. Fig 2. Metabolic degradation of MAB by a homogenate of mouse liver cytoplasm. Disappearing of MAB (*a*). Amounts of metabolized dye at different periods (*b*). Formation of AB (*c*). System as in Fig. 1. The samples contained 0.6 ml of a 10 per cent homogenate.

The binding to protein of C^{14} from MAB was readily demonstrated in the liver homogenates. For this purpose glucose-6-phosphate, fructose-1,6-diphosphate, or isocitrate were included in the system as oxidizable substrates, and TPN as electron carrier. When the mitochondrial fractions were removed from such homogenates, the incorporation properties remained almost unimpaired (Fig. 5). After the removal of the microsomal material, on the other hand, the remaining cell fluid fractions showed a very low

TABLE II

The metabolic degradation of MAB by cytoplasmic fractions from rat and mouse liver.

All fractions added to the samples corresponded to 0.5 ml of a 10 per cent liver homogenate. The systems which contained cell fluid were composed as described under Fig. 1. In the samples with only particulate liver components 2 μM of TPNH was added instead of fructose-1,6-diphosphate and TPN. Incubation time (37°C) 45 minutes. Analytical data expressed in μM.

	Cytoplasmic fractions from rat liver							Mouse liver microsomal fraction
	Total homogenate	Mitochondrial fraction	Microsomal fraction	Cell fluid fraction	Mitochondria + cell fluid	Microsomes + cell fluid	Mitochondria + microsomes + cell fluid	
3 metabolized	0.19	0.02	0.18	0.02	0.03	0.18	0.19	0.10
formed	0.10	0.01	0.09	0.00	0.01	0.09	0.10	0.05

binding activity. When isolated microsomes were resuspended in the isolated cell fluid fraction, the capacity of the system to bind the isotope was almost entirely restored (Table III). The mitochondrial fraction was much less

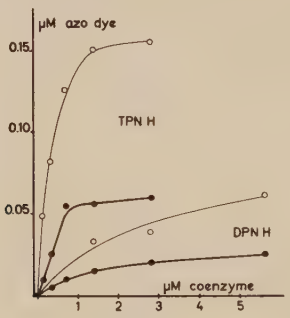

Fig. 3.

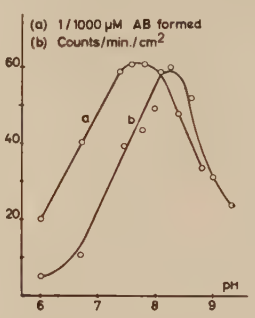

Fig. 4.

Fig. 3. The effects of TPNH and DPNH on the metabolic degradation of MAB by rat liver microsomes. Open circles: total metabolized MAB. Filled circles: AB formed. The incubation system contained in final volumes of 2.0 ml: 120 μM nicotinamide, 10 μM $MgCl_2$, 50 μM potassium phosphate pH 7.6, 100 μM KCl, 0.25 μM MAB, microsomes corresponding to 0.8 ml of a 30 per cent liver homogenate, and indicated amounts of TPNH and DPNH. Incubation time (37°C) 6 minutes.

Fig. 4. pH-curves for (*a*) the demethylation of MAB by rat liver microsomes, and (*b*) the binding of isotope from C^{14}-MAB to microsomal proteins. System as in Fig. 3, with 0.15 μM TPNH per sample. Tris buffer (0.02 *M*) was added in the pH region 7.8–8.5, veronal buffer above 8.5. Incubation time (37°C) 6 minutes. Before the fixation 40 mg of bovine albumin were added to the samples of curve *b* in order to facilitate the preparation of the proteins.

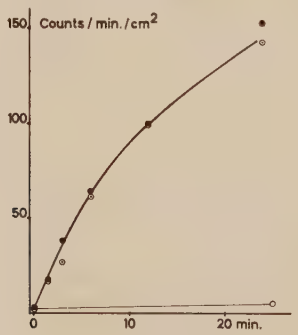

Fig. 5.

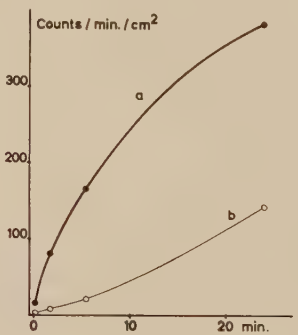

Fig. 6.

Fig. 5. The binding of isotope from C^{14}-MAB to the proteins of rat liver cytoplasm *in vitro*. ● Whole cytoplasmic homogenate; ⊙ Homogenate freed from mitochondria; ○ Cytoplasmic fluid freed from microsomes. System as in Fig. 1, but with 0.25 μM C^{14}-MAB and 0.8 ml of a 30 per cent homogenate.

Fig. 6. The binding *in vitro* of isotope from C^{14}-MAB to the proteins of mitochondria-free rat liver homogenates. Isotope contents at different periods of incubation in (*a*) microsomal proteins, (*b*) cell fluid proteins. System as in Fig. 5, but all constituents added in sixfold amounts to each incubation sample. At the periods indicated the samples were diluted with cold 0.25 *M* sucrose, and the microsomes were isolated by centrifugation, and washed by centrifugation with sucrose.

TABLE III

The formation of C^{14}-labeled protein by the incubation of cytoplasmic rat liver fractions with C^{14}-MAB.

Each fraction was added to the samples in amounts corresponding to 0.6 ml of a 30 per cent liver homogenate. System as in Fig. 5. Each sample was supplemented with the non-included cytoplasmic components at the fixation, in order to obtain equal protein contents in all samples. Incubation time (37°C) 5 minutes.

	c.p.m. per cm^2
Whole cytoplasmic homogenate	49
Cell fluid fraction	3
Cell fluid + mitochondria	5
Cell fluid + microsomes	38
Cell fluid + mitochondria + microsomes	41

TABLE IV

The binding of isotope from C^{14}-MAB to the proteins of different fractions of a rat liver homogenate.

System as in Fig. 5, but all constituents added in sixfold amounts. Incubation time (37°C) 5 minutes.

	c.p.m. per cm^2
Mitochondria	90
Microsomes	192
Cell fluid	44

effective in this respect, and the activity actually observed may partially have been due to microsomal material contamining this fraction.

Homogenates containing mitochondria and microsomes were incubated with C^{14}-MAB under the conditions just described, and were afterwards fractionated by differential centrifugation (Table IV). The highest specific activity of the proteins was constantly observed in the microsomal fraction. A rather high isotope content was usually noticed also in the mitochondrial fraction, while the soluble proteins of the cell fluid had a lower specific activity. In homogenates freed from mitochondria it was observed (Fig. 6) that the initial increase in specific activity was particularly rapid in the proteins of the microsomes while the specific activity of the soluble proteins began to rise after a short initial delay. These experiments suggested that either labeled protein complexes, or reactive metabolic derivatives of the

labeled azo dye were emitted from the microsomes during incubation. In order to arrive at a decision between these two alternatives it seemed desirable to study the binding process in systems with isolated microsomes.

In the presence of TPNH and C^{14}-MAB isotope was readily bound to the proteins of isolated microsomes. When the coenzyme was omitted, no isotope

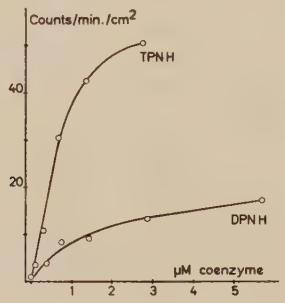

Fig. 7.

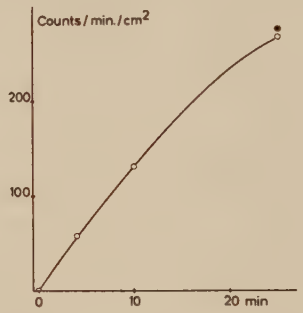

Fig. 8.

Fig. 7. The effects of TPNH and DPNH on the binding of isotope from C^{14}-MAB to the proteins of rat liver microsomes. System as in Fig. 3. Before the fixation 40 mg of bovine albumin were added to the samples in order to facilitate the preparation of the proteins.
Fig. 8. The binding of C^{14} from labeled MAB to the proteins of a mitochondria-free mouse liver homogenate. System as in Fig. 5. The filled circle indicates the incorporation in a similar rat liver system run in parallel.

incorporation was observed. TPNH could be replaced by DPNH only to a limited extent (Fig. 7). The optimal pH-level of the binding process with isolated microsomes and TPNH was 8.0–8.5 (Fig. 4).

A microsomal system was incubated with C^{14}-MAB in the presence of TPNH. After the end of the incubation (25 minutes) the system was chilled in ice, and diluted with cold 0.25 *M* sucrose. Bovine albumin (40 mg) was added as a carrier for soluble proteins. Microsomes and soluble proteins were separated by centrifugation. The isotope content of the proteins in the soluble fraction was low (Table V), indicating that during the incubation only minor amounts of labeled proteins had been released as such from the microsomes. The appearance of labeled proteins in the cell fluid fraction during the incubation of mitochondria-free homogenates with C^{14}-MAB is therefore most easily explained as due mainly to an emission of reactive metabolic intermediates from microsomal elements.

In comparison with the proteins of the cell fluid fraction, bovine albumin incorporated relatively small amounts of activity, even when included in the system from the beginning of the incubation (Table V). The postulated

reactive azo derivatives emitted from the metabolizing microsomes apparently are more easily trapped by the soluble liver proteins than by albumin. The trapping reaction is probably spontaneous, and it takes place with relatively high efficiency even in the cold. Cell fluid samples added after incubation to chilled, diluted microsomal systems incorporated considerable amounts of activity simply during the subsequent isolation in a refrigerated centrifuge (Table V).

TABLE V

The effects of cell fluid proteins and bovine albumin in microsomal systems incubated with C^{14}-MAB.

The incubation samples contained in final volumes of 10 ml: 60 μM glucose-6-phosphate, 1.5 μM TPN, 600 μM nicotinamide, 50 μM $MgCl_2$, 250 μM potassium phosphate pH 7.6, 500 μM KCl, 1.2 μM C^{14}-MAB, glucose-6-phosphate dehydrogenase, and microsomes corresponding to 4 ml of a 30 per cent liver homogenate. Corresponding amounts of cell fluid, or 40 mg of bovine albumin, were added (A) at the start of the incubation, (B) immediately before the centrifugation of the incubated samples (diluted in cold 0.25 M sucrose) in a refrigerated centrifuge (50 minutes at 105,000 g). Time of incubation (37°C) 25 minutes. C.p.m. per cm^2.

	Albumin		Cell fluid	
	A	B	A	B
Microsomes	275	266	296	300
Supernate	25	14	146	68

To explain the binding of azo dyes to liver proteins, reactions of azo metabolites with sulfhydryl groups, or with aromatic side chains of the proteins (Mannich reaction) may be proposed [20, 24, cf. 9, 10]. When glutathione or cysteine were added to microsomal systems metabolizing C^{14}-MAB, a diminished incorporation of the isotope into proteins was observed, possibly due to a competition by the sulfhydryl groups (cf. [24]). Phenylalanine, on the other hand, had no such reducing effect (Table VI). Treatment of the isolated proteins with thioglycollic acid (0.5 M, 90°C for 20 minutes) reduced the specific activity to approximately 40 per cent. It seemed very likely, therefore, that at least some labeled component was bound to the proteins by reaction with sulfhydryl groups.

The addition of AB to a mitochondria-free rat liver system metabolizing C^{14}-MAB reduced the binding of isotope to approximately 60 per cent (Table VI). Since AB thus interfered with the binding reaction, it seemed essential to test C^{14}-AB in similar systems. It was observed that C^{14} from this compound was bound to proteins though generally to a slightly lesser extent than MAB-C^{14}. This observation suggested that the demethylation step was

not of a crucial importance in the binding reactions occurring in these systems. A formation of methylene bridges between the amino group of the azo dyes and aromatic protein residues (Mannich reaction) therefore may not have played a key role in the reactions.

Ribonuclease and protamines, which interfere with the protein metabolism of liver microsomes [1, 16] had no influence on the isotope incorporation *in vitro* in microsomal systems containing C^{14}-MAB (Table VI).

Mitochondria-free mouse liver homogenates showed almost as high capacity for binding C^{14} from MAB as rat liver homogenates (Fig. 8).

TABLE VI

Modified conditions of isotope incorporation. System as in Fig. 5.

Incubation time	Labeled component	Additions	C.p.m. per cm²
5 min.	C^{14}-MAB	–	114
5 min.	C^{14}-MAB	glutathione, 32 μM	51
25 min.	C^{14}-MAB	–	274
25 min.	C^{14}-MAB	cystein, 8 μM	153
25 min.	C^{14}-MAB	cystein, 32 μM	149
15 min.	C^{14}-MAB	–	207
15 min.	C^{14}-MAB	phenylalanine, 30 μM	215
15 min.	C^{14}-MAB	ribonuclease, 0.5 mg	225
15 min.	C^{14}-MAB	clupein, 0.5 mg	215
10 min.	C^{14}-MAB	–	150
10 min.	C^{14}-MAB	AB, 0.25 μM	95
10 min.	C^{14}-MAB	AB, 0.50 μM	91
10 min.	C^{14}-AB	–	99

DISCUSSION

The experiments suggest that constituents of the microsomal material of the liver are intimately involved in the metabolic degradation of azo compounds belonging to the DAB series, and in the binding to liver proteins of atom groups originating from such compounds. There are reasons to believe that reactive oxidation intermediates of the azo dyes are formed in the metabolizing microsomes, and very soon become bound to protein components of these structures [15], possibly by reacting with sulfhydryl groups. The postulated intermediates may soon pass into the cytoplasmic fluid, in which they gradually become trapped by soluble proteins (Fig. 6).

In this process the properties of the trapping protein is a factor of importance. Bovine albumin, when tested in a system of isolated microsomes, was much less effective as a trapping agent than the proteins of the cell fluid. The active component of the cell fluid fraction may be related to the soluble, azo-dye-binding proteins of the liver cytoplasm studied by Sorof *et al.* [27]. The relations between binding capacity and chemical character are being studied at present in experiments with pure microsomal systems, combined with different trapping agents.

It cannot be taken for granted, however, that the isotope incorporation, studied in these experiments, is equivalent to the formation and accumulation *in vivo* of azo-protein complexes during the early stages of tumor induction. The processes actually differ at certain points. A fairly rapid isotope incorporation was observed in experiments *in vitro* with labeled azobenzene, a weakly carcinogenic azo compound [18], which in experiments on tumor induction becomes bound to liver proteins only to a rather limited extent. Isotope from C^{14}-MAB was bound to the proteins of mouse liver homogenates, while the accumulation of bound DAB in mouse livor is rather low in feeding experiments [22]. These divergencies cannot be properly explained at present. It seems possible, however, that differences in reactivity between azo dyes may become more strikingly apparent under the condition of a continual production and breakdown of azo-protein complexes as in feeding experiments, than in the initial stage of the binding reaction. A chemical characterization of the bound isotopic components is intended.

In rats to which carcinogenic azo compounds are administered by way of diet, a steady state may soon be established with respect to the emission of reactive azo derivatives from the submicroscopic metabolic centra and their binding to the proteins of different cell constituents. Under such conditions the cell fluid fraction attains an especially high content of bound azo dye. This fact may be due to an extraordinarily high binding capacity of particular components in this fraction [27].

It has been shown by Glock and McLean [11] that in liver cells triphosphopyridine nucleotide is present to by far the greatest extent in the reduced form. The metabolic decomposition of azo dyes involves a fairly rapid reoxidation of TPNH. Among the enzymatic systems which may become engaged in the reestablishment of the TPNH potential, the enzymes of the oxidative cycle of carbohydrate metabolism are particularly interesting, since they may be indirectly concerned with the metabolism of ribonucleotides [19, 4]. Even if, under normal physiological conditions, ribose phosphate is formed only to a minor extent by a direct oxidation of glucose-6-phosphate [3], the

situation may be different under the condition of a continuous, intensified TPNH-oxidation (cf. [5, 6]). A dietary supply of azo dyes during long periods may be accompanied by an increased metabolic lability of ribonucleotides, and possibly of ribonucleoproteins. Under the influence of potent carcinogenic azo compounds, the specific molecular and structural organization of the nucleoprotein material, and the functions of this material in the production of cell specific proteins may be liable to disturbances by the incessant formation in the submicroscopic structures of reactive azo metabolites, probably with the chemical character of free radicals.

SUMMARY

1. When the carcinogenic azo dyes dimethylaminoazobenzene (DAB) and monomethylaminoazobenzene (MAB), uniformely labeled with C^{14} in the aniline ring, were administered to rats by intravenous injections, a particularly high isotope content was observed in the proteins isolated from the microsomal fractions of the liver.

2. In the presence of TPNH or a TPNH-regenerating system MAB was demethylated *in vitro* by suspensions of liver microsomes. Soluble cytoplasmic proteins had no effect in this reaction.

3. Under the same experimental conditions isotope from C^{14}-MAB became bound to the proteins of the microsomes. The bound isotope was not removed by prolonged extraction with methanol in a continuous extractor, and only to a minor extent by treatment with 5 per cent trichloroacetic acid (90°C). In the absence of reduced coenzyme no binding of isotope was observed.

4. In the demethylation and isotope incorporation experiments TPNH could only to a limited extent be replaced by DPNH.

5. When soluble proteins from the cell fluid fraction were present in microsomal systems metabolizing labeled MAB, an isotope incorporation into the soluble proteins took place after a short delay. When bovine albumin was added to the microsomal systems instead of the soluble liver proteins only minor amounts of bound isotope were observed in the proteins of the medium after the incubation. Without microsomes, however, the soluble liver proteins did not bind any isotope.

6. A metabolic degradation of MAB, and a binding to protein of C^{14} from labeled MAB was observed also in microsomal systems isolated from mouse liver.

7. It is believed that reactive oxidation intermediates, possibly with the character of free radicals, are formed in microsomal units metabolizing

MAB. These postulated intermediates may react with protein components in the same structures, but may soon pass into the cytoplasmic fluid, where they gradually become trapped by soluble protein components.

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ISOLATION OF A FERTILIZATION-PROMOTING FACTOR FROM EGG WATER OF *PSAMMECHINUS MILIARIS*

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It has been known for almost half a century that one or more substances are secreted from the sea urchin egg into the surrounding water. Sea water in which marine eggs have been stored is usually referred to as egg water. Lillie [11] found that spermatozoa upon egg water treatment responded chemotactically, and increased their motility (sperm activation). A third effect was a reversible agglutination of homologous sperматozoa. Lillie believed these reactions to be of great importance in the fertilization process and called the active substance fertilizin. In an attempt to disclose the chemical nature of this substance, Hartmann, Kuhn and their collaborators [8, 9, 10], in their work on *Arbacia lixula*, isolated and described two physiologically active components. Having in view the hormonal character of these substances, Hartmann, Schartau and Kuhn (see Hartmann and Schartau [8]) proposed the name gynogamones, gynogamone I being responsible for chemotaxis and sperm activation, gynogamone II causing agglutination of the spermatozoa. These studies were made on homogenates of whole eggs, but it was never shown that these gynogamones actually were secreted into the sea water. On the other hand it is known that the jelly coat slowly goes into solution in sea water, and jelly solution, in moderate concentration, does in fact cause the same reactions of the spermatozoa, as does egg water treatment.

The effect of egg water and jelly solutions on spermatozoa has been extensively studied, but the main interest so far has been concentrated on the agglutinating component, the chemistry of which has been thoroughly investigated by Vasseur [21]. He has shown that the agent causing agglutination is the jelly substance *per se*, a high molecular acid mucopolysaccharide. In this work on the chemistry and physiology of the jelly coat he has also found that the effect of sperm activation can be separated from that of sperm agglutination. The component responsible for activation is heat resistant (90°C) and retains part of its activity after periodate oxidation, treatments which both render the agglutinating agent inactive. Vasseur and Hagström

[22] and Bielig and Dohrn [1] have further shown, confirming the results of Cornman [4] on egg water, that at least part of the sperm-activating substance in jelly solutions is dialysable. These findings and the earlier observation of Clowes and Bachman [3], that distillates of egg water have a stimulating effect on sperm motility, indicate that in the activation of spermatozoa a low molecular compound might be involved.

Working on the isolation and identification of low molecular substances in culture media of *Penicillium* molds (S. L.), it seemed worth while trying the same procedures in order to elucidate the possible occurrence and chemical structure of any low molecular substance in the egg water. The rather sensitive and objective method of fertilization rate determinations [7, 5], in recording the over-all outcome of sperm-egg encounter, was, in this orientating phase of the investigation, considered to be most appropriate for testing the physiological activity of any isolated substance.

References to the many contributions on the biological activity and chemistry of these so called gamones or fertilizins can be found in the following review articles and monographs: Lillie [12], Tyler [18], Runnström [17], Bielig and von Medem [2], Vasseur [21], Rothschild [15] and Hagström [6].

MATERIAL AND METHODS

Ripe eggs from the Z-form of *Psammechinus miliaris* were used for the preparation of egg water. Fresh ovaries were placed in sea water, and the eggs were separated from the ovarial tissue through fine silk cloth. After one washing the eggs were transferred to a new volume of sea water, where they were kept for 2–3 hours, with occasional stirring. The eggs were then allowed to settle, and the egg water was decanted and filtered through filter paper. Usually 5–15 ml eggs were suspended in 100–200 ml sea water. The sea water, used in the preparations, was filtered and had a salinity of about 24 per cent, the mean value of the salinity at the dredging localities.

The egg water was extracted with petroleum ether (b.p. 40–60°), ether or ethyl acetate by shaking in a separation funnel with an equal volume of the organic solvent (4 times; each time with a ¼-volume of the solvent). The extracts, concentrated by evaporation *in vacuo* or under an infrared lamp, were after application on filter paper, tested with different inorganic and organic reagents. The reagents were: ferric chloride, phospho-molybdic acid, potassium permanganate, bromo-phenolblue, dinitrophenylhydrazine, 2,6-dibromo-quinone-chlorimide and three diazo reagents, diazobenzene-sulphonic acid (D I), 4-benzoyl-amino-2,5-dimethoxy-aniline diazotate (D II) and diazotised *o*-dianisidine (D III). An extensive reference file on the color reactions of these reagents with known substances, as well as their R_f values in six different chromatography systems, have been prepared by Dr. L. Reio in Professor G. Ehrensvärd's group at the Department of Physiological Chemistry. The authors want to express their appreciation of having this, as yet unpublished, reference system at their disposal.

As mentioned above, the method of fertilization rate determination, worked out by Hagström and Hagström [7, cf. 5], was used as a test for the physiological activity of the isolated substances. In some cases a parallel activity test was made by measuring the oxygen uptake of a sperm suspension at 20°C, using the standard Warburg technique. Sperm and eggs from the Z-form were used in these experiments, with the exception that, later in the sea urchin season, some activity tests were made on the S-form, the deep water variety.

RESULTS

Evaporation of ethyl acetate and ether extracts revealed at once the presence of a substance with a yellow green fluorescence in ultraviolet light. Color tests on filter paper gave faint reactions with phospho-molybdic acid, permanganate, dinitro-phenylhydrazine, two of the diazo reagents (II and III) and dibromo-quinone-chlorimide. Comparison of these color reactions with those of the reference substances pointed to the presence of vanillin or *iso*-vanillin in the egg water.

The efficiency of petroleum ether, ether and ethyl acetate as organic solvents was compared by weighing the amounts extracted. 1200 ml egg water, prepared from 60 ml eggs, was evaporated *in vacuo* to 200 ml. Aliquot volumes of pH = 8.30 were shaken, and from 20 ml eggs 3.9 mg, 6.8 mg and 5.4 mg could thus be extracted by petroleum ether, ether and ethyl acetate respectively. In another preparation, 1500 ml egg water of pH = 8.05 from 75 ml eggs was extracted with ether without previous evaporation. 15.4 mg extractable substance, giving a slight smell of vanilla, was obtained in this case.

Evaporation of the extracts to dryness rendered the residue insoluble in distilled water, at least from what could be judged by the fluorescence in ultraviolet light. The residue was, however, soluble in glycerol, and a glycerol solution of 1.9 mg ether extracted substance (from vacuum distilled egg water) in 1.2 ml glycerol was tested for its biological activity. In the fertilization rate experiments 1 drop of "dry" sperm was diluted to 10 ml sea water, of which 1 ml was added, together with the substance under investigation, to 100 ml of egg suspension. The spermatozoa were killed at various intervals by pouring the sperm-egg suspension into a weak solution of sodium lauryl sulphate in sea water. The final concentration of the spermatozoa was about 2×10^6/ml and that of the substance 3.4 and 0.17 gamma/ml. The control was made up with an equal amount of glycerol.

Fig. 1 shows that the egg water substance, in its lower concentration, increases the fertilization rate. When present in the higher concentration, the substance has no fertilization-promoting effect. It has in other experiments,

where the exact amounts of the egg water substance were not known, been shown that e.g. a 5-fold increase in substance concentration gives a decrease in fertilization rate, as compared with the rate recorded for the lower concentration.

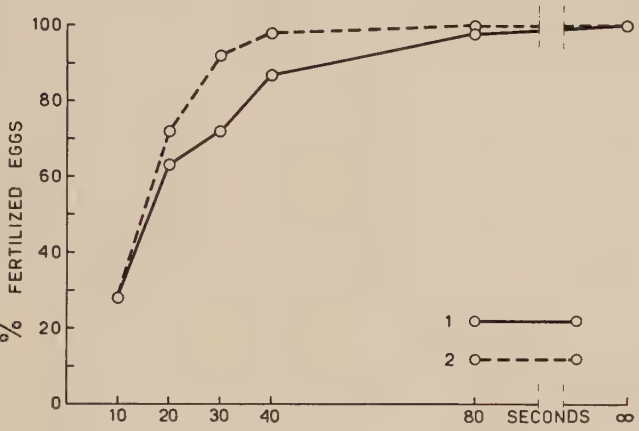

Fig. 1. *1.* Fertilization rate of control eggs. *2.* Fertilization rate of eggs inseminated in the presence of egg water substance in a concentration of 0.17 gamma/ml.

The fertilization was stopped 10, 20, 30 ... seconds after insemination by sodium lauryl sulphate in a final concentration of 0.001 per cent. Each circle of the curves represents about 250 counted eggs.

The substance was also tested for its effect on sperm respiration. The Warburg vessels were charged with 1.5 ml sperm suspension (1:6) in the main compartments and 0.5 ml sea water of different substance concentrations in the side arms.

Fig. 2 gives the oxygen uptake before and after addition of the test substance. It is evident that the presence of glycerol in a concentration of 0.069 molar does not change the rate of oxygen consumption. The egg water substance, in a final concentration of 85 gamma/ml, on the other hand, gives a 6-fold increase in the rate of oxygen absorption, a rate which, still after 2 hours, is 2.5 times as high as the consumption before addition of the substance. In a concentration of 8.5 gamma/ml the substance does not have any effect on the respiration.

In another experiment with a somewhat denser sperm suspension (1:4), 85 gamma/ml of the same egg water substance gives now only a 3-fold increase in the respiratory rate. A slight increase in the rate is obtained with a concentration of 850 gamma/ml, an effect which is accounted for by the presence of glycerol in a concentration of 0.69 molar.

In order to investigate whether vanillin takes an active part in fertilization, the physiological action of this and structurally related compounds were tested with the fertilization rate method. Of the tested substances only vanillin had a promoting action on fertilization. A concentration of 10^{-6} molar was

always found to be effective, whereas higher concentrations either caused a slight decrease or had no effect at all. 10^{-6} molar solutions of guaiacol and piperonal decreased the fertilization rate, and a 1 per cent saturated solution of eugenol in sea water did not interfere with the fertilization process.

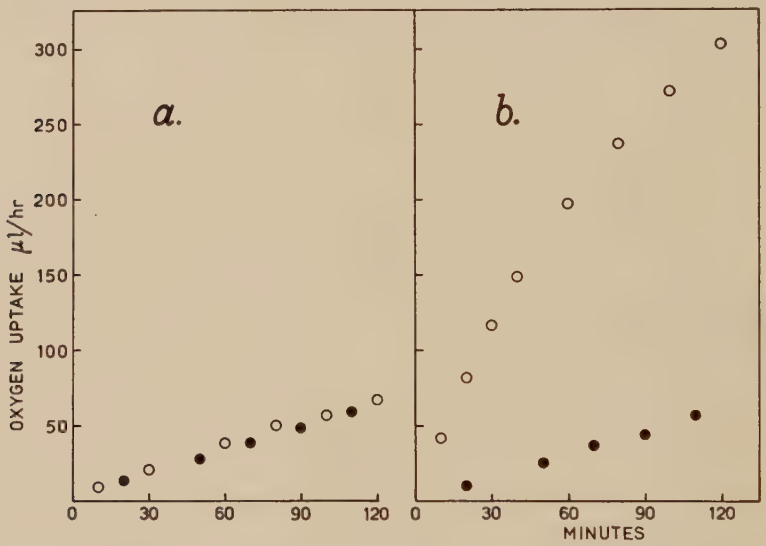

Fig. 2. The filled circles represent the oxygen uptake of a sperm suspension ("dry" sperm diluted with sea water 1:6) before addition of the substance to be tested. The open circles stand for the respiration of the same suspensions after addition of sea water solutions of the test substances. (*a*) represents the oxygen uptake before and after addition of glycerol in a final concentration of 0.069 molar. (*b*) represents the oxygen uptake before and after addition of egg water substance and glycerol in final concentrations of 85 gamma/ml and 0.069 molar respectively.

The influence of vanillin and piperonal on sperm activation was tested for by respiration measurements. The following molarities were used: vanillin, every 10-potence from 2.5×10^{-3} to 2.5×10^{-8}, and piperonal, 2.5×10^{-4}, 2.5×10^{-5} and 2.5×10^{-6}. Addition of vanillin in a concentration of 2.5×10^{-3} molar decreased the rate of oxygen uptake from 29 μl/hr to 17 μl/hr. Piperonal and vanillin in lower concentrations did not change the original respiratory rate of the sperm suspensions.

Using the S-form of *Psammechinus miliaris*, further fertilization rate experiments were made with vanillin, *iso*-vanillin, *o*-vanillin and vanillic acid. To 5 ml of sea water appropriate amounts of the substance were added to give a final concentration of 10^{-6} and 10^{-7} molar. 1 drop of "dry" sperm was added to this mixture, and the sperm and the substance were allowed to interact for 3 mins., whereupon 1 ml of the sperm suspension was used

for insemination of eggs in 100 ml sea water. The substance was thus still present, but the molarities were decreased to 10^{-8} and 10^{-9}, concentrations which were found to be too low to cause any effect. The control was made up in exactly the same way, except for the presence of the substance to be

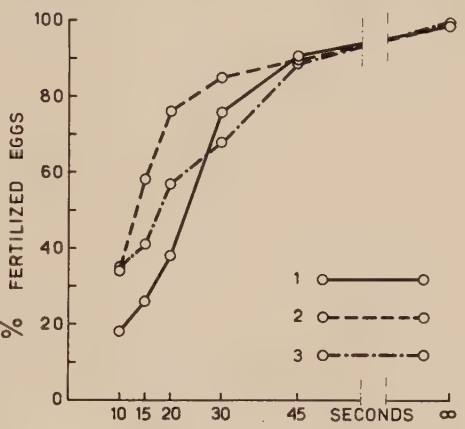

Fig. 3. *1.* Fertilization rate of control eggs. *2.* Fertilization rate of eggs inseminated with spermatozoa pretreated with vanillin for 3 mins. in a concentration of 10^{-6} molar. *3.* Pretreatment in 10^{-7} molar.

The final concentration of sperm was about 4×10^6/ml. Each circle of the curves represents about 250 counted eggs. For further explanations see text.

tested. Similarly these substances were tested for an action on the egg by pretreatment of the egg suspension for 10 mins. in substance concentrations of 10^{-6} and 10^{-7} molar. After washing the eggs were inseminated with untreated spermatozoa.

The fertilization rate curves in Fig. 3 show that pretreatment of the spermatozoa with vanillin has a pronounced effect on the fertilization rate. Compared with the negative results of pretreatment of the eggs, it appears as if the fertilization-promoting effect of vanillin can be ascribed to its action on the spermatozoa. *Iso*-vanillin, *o*-vanillin and vanillic acid do not change the fertilization rate, and it seems as if vanillin is quite specific in its action.

DISCUSSION

As an indication of a physiological action of egg water, jelly solutions or any substance to be tested, many workers have used the rather subjective method of observing the change in sperm motility, a change which is hard to detect and requires the use of aged suspensions with slowly moving spermatozoa. Another test for sperm activation is to follow the oxygen uptake of sperm suspensions, a method giving quantitative information about the effect of the substances under investigation. The influence of egg water on respira-

tion has been studied in many sea urchin species, often with contradictory results. See e.g. Vasseur [20, 21] and Rothschild [14, 15].

In the present investigation the sperm suspensions were always used, in the respiration measurements, within half an hour after dilution. The spermatozoa consumed oxygen at a constant rate for at least 5 hours and had a high motility. Addition of sea water, in the proportion 1:3, during the experiment, did not change the respiratory rate. Addition of egg water substance, prepared by ether extraction of egg water, did, however, in a certain concentration, cause a striking increase in the rate of oxygen uptake (Fig. 2). The concentration of the sperm-activating component may thus be another factor, besides ripeness and age of the spermatozoa, contributing to the diverging results referred to above. It is possible that further studies on sperm respiration may reveal that higher concentrations of the egg water substance will cause a decrease in the respiratory rate.

These two methods, observation of changes in sperm motility and respiration measurements, do not give any information about the importance of the sperm activating substances in fertilization. It is, however, shown that many substances, including egg water and jelly solutions, which increase sperm motility in aged suspensions, also give a higher percentage of fertilized eggs as compared with the controls. (See e.g. [23, 19, 16]. Cf. also [5, 6]). According to Rothschild and Tyler [16, 19], the favourable influence of these substances is supposed to be due to their action as chelating agents for heavy metals, said to have a poisonous effect on the spermatozoa. In a process as complex as fertilization there are certainly many sites for an interaction of various substances.

The egg water substance, used by the present authors in the respiration and fertilization rate experiments, never gave any agglutination of the spermatozoa, but exhibited a sperm-activating effect, recorded as an increase in respiration, and caused further the fertilization to take place with a higher rate.

Vanillin, which was not isolated from the extracted egg water substance, but the presence of which was indicated, did not have any effect on sperm respiration, except for a slight decrease at high concentration. It gave, however, in experiments with both the Z- and S-form of *Psammechinus miliaris*, an increase in the fertilization rate, an effect which seemed to be caused by an action on the spermatozoa.

These findings indicate that, besides the two already known sperm-activating and agglutinating properties of egg water, a third factor, probably vanillin, is involved. The negative results with closely related compounds as

iso-vanillin, *o*-vanillin and vanillic acid suggest that vanillin is quite specific in its action. Unfortunately, vanillyl alcohol was not available at the time of this investigation, but is of interest in this connection, as Clowes and Bachman [3] have found that some alcohols have a stimulating effect on sperm motility.

Metz and Donovan [13] suggested that free amino groups must be present on the surface of the spermatozoa in order to obtain agglutination. Vasseur [21] made the suggestion that the amino groups on the sperm react with sulphate groups in the agglutinating agent. Acidic sulphate groups in the jelly coat can probably also react with positively charged amino groups on the sperm surface and prevent the further penetration of the spermatozoa, unless these positive charges have earlier been neutralized. It does not seem too remote to ascribe to vanillin such a neutralizing effect, and its specificity gives support to this view.

SUMMARY

A substance with a fertilization-promoting effect has been extracted from egg water of *Psammechinus miliaris*.

The presence of vanillin in this egg water substance is indicated by spot tests with several reagents.

The substance has, besides the over-all favourable influence on the fertilization, a sperm-activating effect, recorded as an increase in the respiratory rate.

Vanillin, when tested for its action in the fertilization process, gives an increase in the fertilization rate, but does not cause a higher sperm respiration.

Vanillin seems to be specific in its action and is suggested to function by coupling with positive amino groups on the surface of the spermatozoa.

This investigation has been carried out at the Kristineberg Zoological Station on the Swedish west coast. The authors wish to express their thanks Dr. G. Gustafson, the Director of the Station, and to the other members of the Staff for their kind co-operation and to Mrs. Assia Murray for good help and skilful technical assistance.

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BEHAVIOR OF ENZYMES IN LIVER OF STARVED RATS[1]

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Starvation in rats is known to cause a loss of liver *cytoplasmic* material with a concomitant decrease in cell size [14]. Apparently, rat liver *nuclei* are unaffected by fasting for a period as long as one week [14]. The liver cells of starved rats contain practically no glycogen and have a lower content of water, ribonucleic acid, phospholipide and protein [14, 16]. The relation between the nitrogen (protein) content of liver tissue of starved rats and its enzymatic activity has been examined [16, 20, 22]; but the results are still fragmentary [16]. It has been postulated that the depletion of liver cytoplasmic components in fasting might be related to the loss of morphological units in the cell [20].

The assessment of the biochemical effect of fasting on rat liver tissues has been complicated by the fact that numerous modes for expressing the biochemical data have been used [13]. This problem was discussed in the past [8, 13, 16]. In the present investigation the results were expressed *per nucleus*[3] actually counted in the tissue suspension. This unit was preferred to DNA[4] [8, 16] because the counting of nuclei in liver homogenate is simpler and less time-consuming than the chemical determination of DNA. Otherwise, these two bases for expressing the data are equivalent, since the amount of DNA per liver cell does not change during starvation [14].

The present study of the effect of prolonged period of fasting (seven days) on several liver components was initiated as an extension of studies conducted on hepatoma cells [2, 11]. It was felt necessary to know the effect of fasting on rat liver to help the understanding of the biochemical behavior of hepatoma cells [26].

Determinations of the RNA and nitrogen content were made. The liver cellularity[5] and mitochondriality[5] [4] were determined by actual counts

[1] Aided by grants from the National Cancer Institute of Canada.

[2] Research Associate of the National Cancer Institute of Canada.

[3] The terms "per nucleus", "per average cell" and "per cell" are taken as synonyms.

[4] The following abbreviations are used: P, phosphorus; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; GDHase, glutamic dehydrogenase; RNAase, ribonuclease; Pase, phosphatase; ATPase, adenosinetriphosphatase; G-6-Pase, glucose-6-phosphatase; DPN, diphosphopyridine-nucleotide.

[5] The terms cellularity and mitochondriality were defined previously [3].

of nuclei and mitochondria in the tissue homogenate. The activities of eleven enzymes related to various metabolic reactions of rat liver were studied.

Results of this study showed that starvation of seven days caused an increased liver cellularity and no change in the liver mitochondriality. The nitrogen and RNA content, the number of mitochondria, and several enzymatic activities were decreased, per average cell, to about 50 per cent the value of fed animals. One enzyme activity was the same in liver of fed and fasted rats whereas a few enzyme activities decreased slightly. One enzyme fell to extremely low level of activity in liver of fasted rats. A general correlation between the actual loss of cytoplasmic organelles and enzymatic activity in liver of fasted animals was not demonstrated.

MATERIALS AND METHODS

Male Wistar rats of an average weight of 230 grams were placed in separate cages. The control group was fed Purina Fox Chow and tap water *ad libitum*. The other group received only water. Animal weights were registered at the start and termination of the experiment. The animals were starved for seven days and both groups were then sacrificed by stunning and decapitation. The livers were excised, blotted on filter papers and placed in a beaker standing on ice. The cooled livers were weighed and a pulp was prepared by passing the tissue through a Plexiglas press[1] [4]. The fibrous material adhering to the sieves was discarded and an exact amount of tissue pulp was weighed to prepare a ten per cent homogenate in 0.88 *M* sucrose. Homogenization was carried out in the cold for 2½ minutes at 600 rpm using a Teflon pestle. A similar homogenate was prepared in physiological saline and employed for nuclear counts as described recently [4].

The following enzymes were assayed: GDHase, guanase, alkaline and acid RNAases, acid and alkaline Pases, uricase, inosine phosphorylase, ATPase, cathepsin and xanthine oxidase. These enzyme activities were determined as described in Table I. Proportionality with time of incubation and concentration of tissue homogenate when assayed at optimal concentration of substrate was verified for each enzyme appearing in Table I. Nitrogen was determined by the micro-Kjeldahl procedure and RNA by the method of Ceriotti *et al.* [7]. Mitochondria were counted in the liver homogenates by a technic previously described [5].

RESULTS

Liver constituents.—The effect of fasting on various rat liver components is shown in Table II. These results demonstrated that 7 days' fasting caused a 54 per cent decrease in liver weight and 22 per cent in the liver/body

[1] Devised by one of us (C. A.) and available commercially at Hickey Plastic Co., 1898 St. Antoine Street, Montreal, P. Q. Canada.

TABLE I

Details on enzymes assay procedures.

Enzymes	GDHase [15][1]	Guanase	Alkaline RNAase [12]	Acid RNAase [12]	Acid Pase [3]
Substrate and other factors; final concentration	L-glutamate: 0.013 *M*; DPN·$4H_2O$ (California Foundation): 0.0036 *M*; Nicotinamide: 0.03 *M*; KCN: 0.004 *M*	Guanine: 6.7×10^{-5} *M*; KCN: 2×10^{-4} *M*; xanthine oxidase (Worthington): in excess	Yeast RNA (Schwarz): 0.35 %	Yeast RNA (Schwarz): 0.075 %; $MgSO_4$: 0.01 *M*	β-Na glycerophosphate: 0.08 *M*
Buffer; final concentration and pH	Phosphate: 0.04 *M*; pH 7.2	Phosphate: 0.15 *M*; pH 7.5	Acetate-borate-cacodylate: 4.5×10^{-4} *M*; pH 7.5	Acetate-borate-cacodylate: 4.5×10^{-4} *M*; pH 5.5	Acetate-acetic-acid 0.3 *M*; pH 4.5
Volume, ml, final	3	3	4	4	6
Incubation time, min, T°C	5; room T°	6; room T°	30; 37°	30; 37°	60; 37°
Units of activity, definition	Micromoles DPN reduced/min/100 mg fresh tissue	Micromoles of uric acid formed/min/100 mg fresh tissue	Arbitrary units: increase in O.D. at 260 mμ of the acid soluble following acidification with $HClO_4$; calculated for 1 g fresh tissue	Arbitrary units: increase in O.D. at 260 mμ of the acid soluble following acidification with $HClO_4$; calculated for 1 g fresh tissue	Gamma of inorganic P liberated/hr/100 mg fresh tissue
Remarks	Homogenate frozen at –20°C and thawed to release latent activity [15]	U.V. readings at 292.5 mμ			Homogenate preincubated 1 hr at 37°C to liberate most of the latent enzyme activity [3]

[1] Numbers in brackets refer to methods outlined in the literature.

Table I (*continued*).

Enzymes	Alkaline Pase [4]	Uricase [23]	Inosine phosphorylase [23]	ATPase [21]	Cathepsin [1]	Xanthine oxidase
Substrate and other factors; final concentration	β-Na glycerophosphate: 0.1 M	Uric acid: 4.2×10^{-4} M	Inosine: 3.3×10^{-4} M; xanthine oxidase (Worthington): in excess; KCN: 2×10^{-4} M	ATP (Schwarz); chromatographically pure: 2×10^{-3} M; $MgCl_2$: 2×10^{-3} M	Hemoglobin: 3.9×10^{-4} M	Xanthine: 3.9×10^{-4} M
Buffer; final concentration and pH	2-amino-2-methyl-1,3-propanediol: 0.033 M; pH 9.8	Phosphate: 0.15 M; pH 7.5	Phosphate: 0.15 M; pH 7.5	Tris:[1] 0.5 M; pH 7.4	Acetate-acetic acid: 0.167 M; pH 3.6	Phosphate: 0.15 M; pH 7.5
Volume, ml final	6	3	3	2	3	3.4
Incubation time, min; T°C	60; 37°	5; room T°	6; room T°	15; 37°	30; 37°	15; 37°
Units of activity, definition	Gamma of inorganic P liberated/hr/100 mg fresh tissue	Micromoles of uric acid destroyed/min/100 mg fresh tissue	Micromoles of uric acid formed/min/100 mg fresh tissue	Gamma of inorganic P liberated/15 min/100 mg fresh tissue	Micromoles of tyrosine equivalent formed/min/100 mg fresh tissue	Micromoles of uric acid formed/min/100 mg fresh tissue
Remarks	This method measures the Mg^{++} insensitive enzyme only [3]	U.V. readings at 292.5 mμ	U.V. readings at 292.5 mμ	Homogenate preincubated 1 hr at 37° to release latent activity [21]	Homogenate preincubated 1 hr at 37° to release latent enzyme	Supernatant obtained after centrifuging the homogenate at 100,000 g for 1 hr was used to get rid of uricase bound to the sediment. This supernatant was preincubated 40 min at 37°C

[1] Tris (hydroxymethyl) aminomethane.

TABLE II

Effect of 7-day fasting on liver components of the rat.[1]

Components	Fed controls Wet weight	Fed controls Units/nucleus	Fasted Wet weight	Fasted Units/nucleus	Per cent loss per nucleus
itial animal weight, g	230 ±2		228 ±2		
inal animal weight, g	264 ±5		148 ±9		
inal liver weight, g	8.9 ±0.4		3.9 ±0.7		
inal liver/body weight ratio × 100	3.37 ±0.25		2.64 ±0.38		
ellularity: nuclei/g × 10^{-6}	204 ±40		394 ±74		
itochondriality: Mitochondria/g × 10^{-10}	31.1 ±1.6	1525	32.2 ±2.9	817	46
NA, mg/g	7.3[2]	35.8 × 10^{-9}	6.8	17.3 × 10^{-9}	52
itrogen, mg/g	31.9 ±0.5	156.0 × 10^{-9}	38.8 ±1.4	98 × 10^{-9}	37
DHase	0.291 ±0.032	14.4 × 10^{-9}	0.495 ±0.076	13.8 × 10^{-9}	4
uanase	0.083 ±0.006	4.12 × 10^{-9}	0.127 ±0.007	3.31 × 10^{-9}	20
lkaline RNAase	680 ±202	3.33 × 10^{-6}	1109 ±25	2.81 × 10^{-6}	16
cid RNAase	338 ±68	1.66 × 10^{-6}	504 ±91	1.28 × 10^{-6}	23
cid Pase	1122 ±157	55.0 × 10^{-6}	1624 ±250	41.2 × 10^{-6}	25
lkaline Pase	164 ±60	8.0 × 10^{-6}	213 ±52	5.4 × 10^{-6}	32
ricase	0.111 ±0.019	5.71 × 10^{-9}	0.110 ±0.008	2.89 × 10^{-9}	49
ıosine Phosphorylase	1.96 ±0.15	99.6 × 10^{-9}	1.80 ±0.13	50.0 × 10^{-9}	50
TPase	1472 ±120	72.1 × 10^{-6}	1418 ±108	36.9 × 10^{-6}	49
ıthepsin	0.096 ±0.040	4.7 × 10^{-9}	0.089 ±0.018	2.26 × 10^{-9}	52
anthine oxidase	0.018 ±0.005	0.86 × 10^{-9}	0.004 ±0.001	0.100 × 10^{-9}	88

[1] Mean and S.D. of four pools of four livers.
[2] One determination on one pool of four livers.

weight ratio. A twofold increase in liver cellularity was observed in fasted animals, whereas the mitochondriality remained the same. The number of mitochondria per average cell was decreased in the liver of starved rats to half of the value of fed animals. These results confirm and extend previous data from this laboratory [4, 24]. The RNA content per cell in the fasted rats was 50 per cent of the value in fed animals. The total nitrogen[1] content per cell in liver of fasted rats was decreased to about 40 per cent of the fed values. These data agree well with recent observations of Laird *et al.* [17].

[1] The expression of the nitrogen content per average cell was used here, bearing in mind that nitrogen is of intra- and extracellular origin.

Liver enzymes.—Table II shows that in 7-day fasted rats, uricase, inosine-phosphorylase, ATPase and cathepsin activity decreased per average cell to 40–50 per cent the level of activity observed in fed animals. The enzymes guanase, acid and alkaline RNAases, acid and alkaline Pases were decreased in activity in fasted rats to a level corresponding to 65–75 per cent of the level observed in fed rats. Xanthine oxidase was decreased to 11 per cent of the level found in fed animals. On the other hand, GDHase activity was not affected by fasting since the activity per average liver cell was the same in fed and starved rats.

DISCUSSION

The results on the effect of a 7-day fasting on acid Pase, RNAases and alkaline Pase extend our previous data on 1 day of fasting [3]. The decrease in alkaline Pase activity (the Mg^{++} insensitive) observed in liver of starved rats was parallel to the decrease in nitrogen content of liver homogenate. This confirm results of previous investigators [16, 20, 22].

The liver cellularity in 7-day starved rats was shown to be twice that of fed animals. This is mainly due to the known phenomenon of liver shrinkage occurring during fasting [14]. A twofold increase in cellularity suggests a loss of half the mass of liver cell cytoplasm, since the nuclear mass is negligible as compared to the whole cell mass [13]. Therefore, an actual 50 per cent loss of any components in the average liver cell would simply reflect a general loss of liver cytoplasm. For instance, the decrease in the number of mitochondria and RNA content per liver cell of fasted rats represents such a non-preferential loss of liver components. Similarly, a loss of uricase, inosine phosphorylase, ATPase and cathepsin activity in the average liver cell of 7 days starved rats is suggested as being a non-preferential loss of specific protein which was proportional to a general loss of cytoplasm.

On the other hand, the decrease in xanthine oxidase activity in liver cells of fasted rats exceeded the loss of the components just mentioned. This marked depletion of xanthine oxidase is taken as an indication of a specific effect of starvation on that particular enzyme. It has been known that liver xanthine oxidase activity is much depressed in fasting [16, 19, 27, 28, 29, 30], but the significance of the phenomenon is not understood [16]. It is of interest to mention that among the purine metabolizing enzymes studied here (inosine phosphorylase, guanase, xanthine oxidase and uricase) only the enzyme xanthine oxidase was depressed in activity to a level lower than 50 per cent of the values observed in fed animals. For instance, guanase activity

was maintained at a high level in liver of fasted rats, whereas inosine phosphorylase and uricase activities were decreased per cell to 50 per cent the level found in fed animals. The maintenance of high activity of liver guanase and very low activity of xanthine oxidase is of interest in view of their position in the sequence of reactions leading to uric acid formation. The respective level of activity of guanase and xanthine oxidase in liver of fasted rats suggest that the rate of catabolism of xanthine might be so controlled as to maintain the level of xanthine in fasted animals. However, a residual liver xanthine oxidase activity in fasted rats of about 10 per cent of the fed value might be sufficient to metabolize the amount of xanthine actually reaching the liver cells of fasted rats.

The maintenance of high level of both acid and alkaline RNAases, acid Pase and GDHase emphasized the fact that the residual cytoplasm of liver of starved rats can be preferentially rich in some components. It is of interest to observe that the RNA content per average liver cell markedly decreased in the presence of a maintained RNAases activity. The maintenance in starvation of high level of liver acid phosphatase activity but not of cathepsin and uricase is of particular importance since these three enzymes are bound to the same class of cellular particulates known as the "lysosomes" [8, 9]. Uricase is strictly an insoluble enzyme, i.e., an architectural component [9]. It is difficult to understand a loss of an architectural protein like uricase without a concomitant loss of acid Pase and cathepsin, if one assumes that all three enzymes are bound to the same morphological units.[1] The behavior of these three enzymes in fasting would rather support the concept that the "uricase granules" belong to a class of rat liver particulate, different from the "lysosomes" [9, 10].

The maintenance of GDHase activity per average cell following inanition for 7 days is also of interest from a cytochemical point of view. GDHase is concentrated in liver cell particulates and mainly in mitochondrial elements [9, 14]. It has been shown here that the number of mitochondria in liver cells of fasted rat was decreased to 50 per cent of the number found in liver of fed animals. Nevertheless, the GDHase activity was shown to be maintained per cell. This fact indicates that liver mitochondria of 7-day starved rats contain more GDHase activity than the corresponding particulates of fed animal. Furthermore, it was previously demonstrated that liver G-6-Pase activity which is of *microsomal origin* was increased in 1- and 3-day fasted rats and mice [5, 17, 24] when expressed per wet weight. It was also shown

[1] Allard, C. and de Lamirande, G., unpublished data showed that the intracellular distribution of acid Pase, cathepsin and uricase is the same in liver of fed and 7 day starved rats.

that liver G-6-Pase activity was maintained on normal levels in the average cell after 24-hour fasting [24]. These findings have recently been extended by showing that hepatic G-6-Pase activity was maintained in the average liver cell on normal levels even after a 7-day fast in the rat, despite the fact that the RNA content (mostly of microsomal origin [10]) was decreased by 50 per cent. The behavior of G-6-Pase during fasting indicate that microsomes of 7-day starved rats contain more G-6-Pase activity than the corresponding particulates of fed animals.

The maintenance of GDHase activities in liver cells of fasted rats is of particular interest in view of its possible role in the physiology of the animal. In fasting, the only source of blood glucose comes from non-carbohydrate precursors through gluconeogenesis. Since the enzyme GDHase catalyses specifically the transformation of glutamate into α-ketoglutarate, the maintenance of this enzyme activity in the liver cells of starved rats might permit an unimpaired gluconeogenesis via the glutamate pathway. Unpublished data from this laboratory showed that in 18 hour fasted hypophysectomized animals the liver GDHase activity per average cell was markedly depressed.[1] It is known that the process of gluconeogenesis is nearly abolished in hypophysectomized animals. Thus, the maintenance of liver GDHase activity per cell in fasting, and its lower activity in hypophysectomized animals support our hypothesis that the level of GDHase activity in liver might be an indication of the rate of gluconeogenesis from protein.

SUMMARY

The purpose of these experiments was to study the effect of prolonged fasting on various rat liver enzymes and correlate these biochemical effects with changes in cellularity and amount of various cytoplasmic components.

It was observed that a 7-day fast caused a loss of liver cytoplasmic components. These data suggested that on one hand, several liver constituents (mitochondria, ribonucleic acid, and adenosinetriphosphatase, uricase, inosine phosphorylase and cathepsin activities) decreased in amount or activity in fasting in proportion with a loss of liver cytoplasm. On the other hand, glutamic dehydrogenase as well as acid and alkaline ribonucleases, acid phosphatase and guanase, were maintained at high level of activity per average liver cell of starved rats. Among the purine metabolizing enzymes investigated, xanthine oxidase activity was preferentially depleted in the liver

[1] Allard, C. and Cantero, A., unpublished observations.

of fasted animals. The latter results were discussed in relation with the metabolism of purines.

The maintenance of glutamic dehydrogenase activity in liver cells of fasted rats was discussed in view of its possible role in gluconeogenesis.

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SYNTHETIC PROCESSES IN NUCLEATED AND NON-NUCLEATED PARTS OF *MYTILUS* EGGS

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The role of the nucleus in the synthetic processes in cells has been debated for very many years. Recently several authors approached the problem anew by following the rate of incorporation of radioactively labelled tracers into cells or parts of cells from which the nucleus had been removed in some way. A recent review of the literature will be found in Brachet's article in *Recent Developments in Cell Physiology* [4]. His own experiments and those of his collaborators have, in the main, dealt with the synthetic processes in fragments of the alga, *Acetabularia mediterranea* or of *Amoeba*, which have been cut away from the main cell in such a way as to remove their contact with the nucleus. Their results show that protein synthesis can continue for a considerable time in the enucleated fragment. After some time, these fragments show a fall in their content of RNA and thereafter the rate of protein synthesis declines sharply. These facts are scarcely compatible with any theory (e.g. that of Caspersson, [5]) which suggests that the nucleus has a direct effect on protein synthesis. Considering its great importance, however, it seems desirable that this conclusion should rest on a wider factual basis relating to different types of organisms and cells. The synthesis of certain proteins such as haem in non-nucleated red blood cells (reticulocytes) provides one further example [12]. Another material in which the problem can be easily investigated is provided by those spirally cleaving eggs, which exhibit a well-marked polar lobe, such as those of *Mytilus*.

TECHNIQUE AND EXPERIMENTS

Specimens of *Mytilus edulis* were collected from the sea-shores of Musselburgh, near Edinburgh. Viable eggs and sperms were obtained by allowing the animals to spawn in individual finger bowls or crystal basins containing sea water, at room temperature.

The eggs obtained were artificially fertilized with the sperm from another bowl. After fertilization the vitelline membrane was dissolved by the lysin of sperm extract (method of Berg [1]). The process of dissolution takes from 5 to 10 minutes. After

the removal of the vitelline membrane, the eggs were washed with calcium-free sea water, prepared as described by McClendon [13] and Robertson and Webb [14].

Shortly before cleavage, a cytoplasmic bulge, the 'polar lobe' makes its first appearance. In development it remains united with the main body of the egg. In the present experiments, the polar lobes were artificially detached from the eggs by treating them with an hypertonic solution following the method described by Berg [2]. The most effective hypertonic medium in our hands was a 0.7 *M* solution of sodium chloride. Owing to the absence of calcium in the medium not only do the polar lobes become detached but also the blastomeres AB and CD of the two cell stage become separated.

The treatment, lasting about one hour, results in the separation of from 50 to 75 per cent of the polar lobes.

The eggs (whole eggs, separated blastomeres and polar lobes) were then transferred to 10 cc of sterile sea water to which 15 μC of an isotope was added. Three such experiments were set up in which the isotopes were respectively, DL-methionine-S-35, glycine-C-14 and adenine-C-14 (supplied by the Radiochemical Centre, Amersham).

The 'eggs' were left in this solution for 2½ hours, after which they were washed in sea water and fixed in Bouin. Dehydration was carried out in alcohols up to 95 per cent, clearing in methyl benzoate and benzene, and embedding in hard wax containing ceresin and stearic acid. A whole pipette-full of 'eggs' was embedded in one block.

Sections of the blocks were made at 5 μ and, from these, autoradiographs were obtained by the technique of Doniach and Pelc [6]. The exposure was prolonged to 14 days.

RESULTS

The majority of the sections in the slides are either of nucleated cells (AB or CD blastomeres) or of non-nucleated detached polar lobes. The latter can be recognised not only by the absence of the nucleus but also by the character of the cortex. It is, however, possible also to find a few one-cell stages from which the polar lobe has not been detached and can still be recognised. There are also a few examples of two-cell stages in which the AB and CD blastomeres have remained in contact with each other and with the polar lobe materials still incorporated into the CD cell. Counts have been made over the few such specimens which could be found in the slides treated with methionine. A few counts of detached polar lobes in the neighbourhood of the one- and two-cell eggs counted are also given in Table I. It will be seen that at the one-cell stage there is very little noticeable difference of uptake between the polar lobe and the remainder of the egg; nor is there any significant difference between the rate of incorporation of methionine into the AB and CD cells. The incorporation is, however, considerably lower in the detached polar lobes.

The same conclusion emerges more strongly from the main bulk of the

TABLE I

Methionine uptake by Mytilus *polar lobes and blastomeres.*
(Grain counts above background per unit area of 54μ^2.)

Detached lobes	One-cell		Two-cell	
	Polar lobe	Rest of egg	AB	CD
5.3	9.4	11.3	17.3	13.2
4.1	11.8	12.0	7.8	9.0
5.3	15.8	16.0	8.0	7.8
4.8	7.8	7.8	10.8	10.2
1.8	7.3	8.0	11.5	10.4
Mean 4.3	10.4	11.0	11.1	10.1

TABLE II

Isotope uptake by Mytilus *detached polar lobes and nucleated blastomeres.*
(Grain counts above background per unit area of 54 μ^2. Each value is the mean of about 50 counts.)

Isotope	Detached polar lobes	Nucleated cells
DL-Methionine-S-35	3.6±2.1	8.8±2.8
Glycine-C-14	1.3±1.1	6.7±4.1
Adenine-C-14	1.5±1.2	4.3±1.3

experimental results in which counts were made over isolated, non-nucleated polar lobes and over nucleated AB or CD cells which were not distinguished from one another. There was considerable variability and although a large number of eggs and fragments were counted, the standard deviations of the means remain high. The experiments with all three tracers, however, are consistent in showing a considerably greater incorporation into the nucleated AB and CD cells than into the non-nucleated polar lobes. (Table II and Fig. 1).

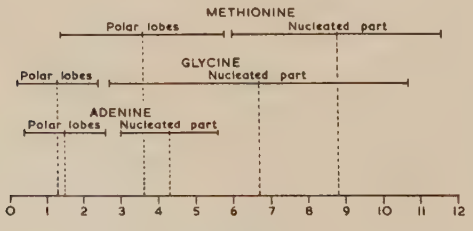

Fig. 1.—Diagram illustrating the range of activity counts per 54 μ^2 in the cell body and the polar lobe (data of Table II).

DISCUSSION

Of the tracers which have been used in this study, methionine will be incorporated into protein, adenine mainly into nucleic acids, and glycine into both. The uptake of all three is reduced in polar lobes which are deprived of their contact with the nucleus. The nucleus, therefore, seems to play an important role in this form in the synthesis of both the proteins and the nucleic acids. We have already briefly mentioned the evidence for the importance of the nucleus in connection with protein synthesis. As regards its functions in connection with nucleic acids, one may recall the demonstration by Brachet and his collaborators that the RNA content of non-nucleated fragments of *Acetabularia* and of Amoebae begins to fall after some time [3]. Goldstein and Plaut [7] have recently provided a very clear demonstration that, in Amoeba, RNA may be manufactured in the nucleus and passed thence into the cytoplasm. Thus a reduction in the uptake of tracers by non-nucleated polar lobes is what the previous evidence would lead us to expect. It is, however, perhaps somewhat surprising to find how rapidly this effect becomes manifest. The eggs in these experiments were treated for a period of only 2 ½ hours and in this short period the effect of the nucleus was clearly to be seen. In *Acetabularia* and even Amoeba on the other hand, the removal of the nucleus seems to remain without effect for a much longer period. The reasons for this difference are not clear. There is, however, some evidence which suggests that the cytoplasm of mature eggs contains comparatively small numbers of microsomes and that these increase in concentration considerably during the early stages of development. This seems to be the case for instance in echinoderms (cf. [8]) and in amphibia (cf. [2, 9, 10, 11]). Since the synthetic activity which continues in parts of Amoeba and *Acetabularia* removed from the nucleus is probably carried out at or by the microsomes, it may be that the comparative dearth of such bodies in the cytoplasm is the reason why the separation of the polar lobe from the nucleus has such a rapid effect in *Mytilus* eggs.

SUMMARY

Artificially fertilized eggs of the sea water mussel, *Mytilus edulis*, were treated with hypertonic sea water and calcium-free sea water in such a way as to cause the polar lobes to constrict off from the remainder of the egg. Batches of eggs from which most of the polar lobes had been removed in this way were allowed to absorb glycine-C-14, adenine-C-14 and methionine-

S-35 for a period of 2½ hours. Autoradiographs were then prepared by the stripping film method and the intensity of uptake estimated by counting activity grains above the sections. For all three tracers, separation of the polar lobe cytoplasm from the nucleated part of the cell led to a considerable decrease in the incorporation of these substances into insoluble materials. In a small series of observations no evidence was found that the polar lobe material has a lower rate of incorporation than the rest of the cytoplasm also when it is still attached to the egg. Similarly no difference of uptake was found between complete AB and CD blastomeres.

The authors are indebted to Professor C. H. Waddington for suggesting this problem, and to the Egyptian Government (A. W) and the Melville Trust (E. P.) for Research Fellowships.

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THE INCORPORATION *IN VITRO* OF LABELED AMINO ACIDS INTO THE PROTEINS OF REGENERATING RAT LIVER

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As is well known, a condition of rapid, hyperplastic growth is initiated in liver tissue as a response to partial hepatectomy [4]. For the study of the metabolic background of growth induction and growth regulation the regenerating liver constitutes a material of unusual convenience.

In isotope experiments *in vivo* it has been observed that the period of rapid regenerative growth is correlated with an increased incorporation of labeled amino acids into the liver proteins [3]. In order to arrive at a more detailed characterization of the protein metabolism during the course of the regeneration cycle it seemed desirable to study the incorporation of amino acids in cell-free systems under varied experimental conditions.

The experiments described in this first report show that the enzymatic systems concerned with the incorporation of labeled amino acids into proteins become activated during the early stages of regeneration. This increased activity involves the amino acid activating enzymes in the cell fluid as well as the incorporation mechanisms in the microsomal structures (cf. [5, 6]). The activation is more pronounced, however, in the microsomal systems.

EXPERIMENTAL

Male albino rats weighing approximately 250 g were used in the experiments. Partial hepatectomy was performed essentially by the method of Higgins and Anderson [4], the median lobe, left lateral lobe, and posterior lobule of the right lateral lobe being removed under ether anesthesia. The animals were maintained on a standard diet before, as well as after the operations. Before the isotope incorporation experiments they were kept without food for 15–20 hours [7].

At different periods after the operations the animals were killed by decapitation. The livers were rapidly excised and were carefully minced in an ice-cold medium containing 0.25 *M* sucrose, 0.025 *M* KCl, 0.035 *M* $KHCO_3$, 0.02 *M* potassium phosphate, pH 7.8, and 0.01 *M* $MgCl_2$ (cf. [10]). After repeated washing the minced livers were homogenized in a loose-fitting glass homogenizer with 2.5 volumes of the same medium. The homogenates were centrifuged for 5 minutes at 12,000 $\times g$ (0°C), and

the upper parts of the supernates were collected by means of a pipette. The lipid layer, which especially in the case of the regenerating livers accumulated at the surface of the supernates, was avoided.

In some experiments the microsomal and cell fluid fractions were separated by centrifugation at 105,000 $\times g$ for 45 minutes. The upper part of the supernates was collected, and the microsome pellets were drained and carefully washed with the homogenization medium. The microsomes were then suspended by gentle homogenization in the same medium. The microsome suspensions were diluted with the medium to 1/6 of the volume of the original mitochondria-free homogenates.

Of the homogenates 0.7 ml samples were incubated at 37°C in final volumes of 1 ml with 0.02 μM of C^{14}-L-leucine (6 mc/mM), or 0.35 μM of C^{14}-glycine (1.44 mc/mM) in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. In the experiments with isolated microsomal and cell fluid fractions 0.65 ml of cell fluid and 0.1 ml of suspended microsomes were incubated under similar conditions. Phosphoenolpyruvate (10 μM) and ATP (1 μM) were included in the incubation systems as the energy source. Phosphoenolpyruvate was synthesized from epichlorohydrin according to Baer [1]. ATP was a Sigma preparation.

The samples incubated with C^{14}-L-leucine were treated with unlabeled L-leucine in large excess, and the proteins were precipitated with trichloroacetic acid (TCA). The protein samples were purified by the method of Schneider [8]. The purified proteins were plated, and the specific activities of the protein samples were recalculated for indefinite sample thickness (1 cm^2 plate area) on the basis of an empirical activity saturation curve.

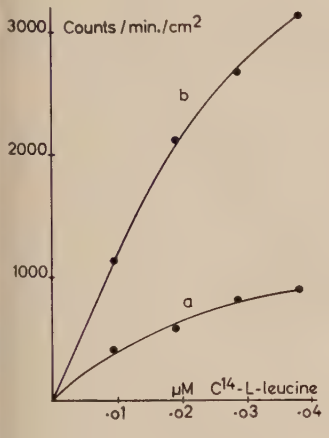
To the samples incubated with C^{14}-glycine unlabeled glycine in large excess was added together with 20 mg of glutathione (Sigma). The samples were precipitated with TCA. From the TCA-extracts glutathione was isolated as a cadmium salt and afterwards crystallized as copper mercaptide [9]. The mercaptide was combusted to $BaCO_3$, and the specific activity of the carbonate was determined. The TCA-precipitated proteins of the same experiments were exhaustively treated with 10 per cent thioglycollic acid. The protein samples were then prepared in the same way as in the experiments with C^{14}-L-leucine. The specific activities were determined and recalculated for indefinite sample thickness as was described previously.

RESULTS AND DISCUSSION

The experiments illustrated by Figs. 1 and 2 show that mitochondria-free homogenates from regenerating rat liver utilized the labeled amino acids more effectively for incorporation into proteins than similar homogenates prepared from normal liver. This effect could not be ascribed to an increased longevity of the homogenates from the regenerating livers under the conditions of incubation, since the rate of incorporation was higher in these homogenates from the very beginning of the isotope incubations (Figs. 2, 4).

It is a general experience that liver regeneration in the rat is subject to a considerable biological variability. In our material the activation of the

amino acid incorporation had reached a maximum about 40 hours after the operations. The activity of the homogenates at the culmination period was 2.5–3 times higher than in the controls (Figs. 2, 4).



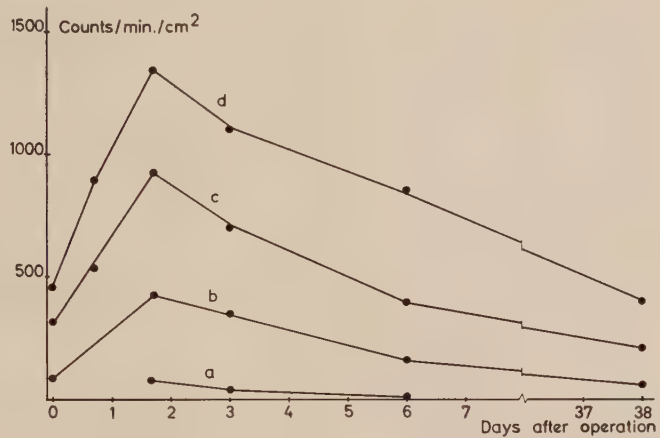
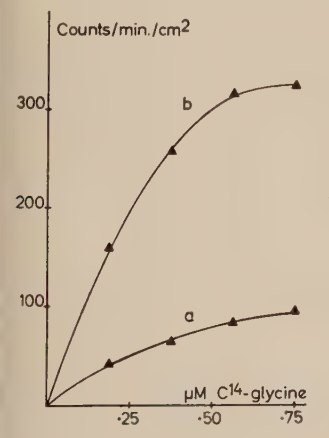


Fig. 1 (*left*).—Incorporation of C^{14}-L-leucine into the proteins of mitochondria-free rat liver homogenates prepared from (*a*) normal liver, (*b*) regenerating liver (40 hours). Effect of the concentration of C^{14}-L-leucine (μM/ml).

Fig. 2 (*right*).—Incorporation of C^{14}-L-leucine into the proteins of mitochondria-free rat liver homogenates at different periods after partial hepatectomy. Incubation (37°C) for (*a*) 1 min., (*b*) 2½ min., (*c*) 5 min., (*d*) 10 min.



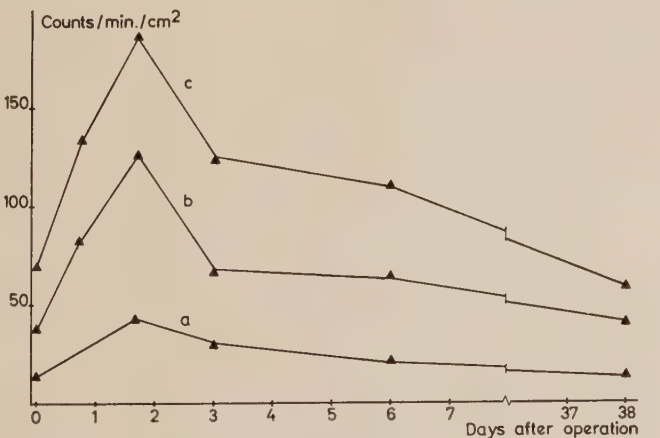


Fig. 3 (*left*).—Incorporation of C^{14}-glycine into the proteins of mitochondria-free rat liver homogenates prepared from (*a*) normal liver, (*b*) regenerating liver (40 hours). Effect of the concentration of C^{14}-glycine (μM/ml).

Fig. 4 (*right*).—Incorporation of C^{14}-glycine into the proteins of mitochondria-free rat liver homogenates at different periods after partial hepatectomy. Incubation for (*a*) 2½ min., (*b*) 5 min., (*c*) 10 min.

The increased rate of amino acid incorporation into proteins during the regeneration cycle was accompanied with an increased incorporation of C^{14}-glycine into glutathione (Figs. 5, 6). Time course experiments (Fig. 6)

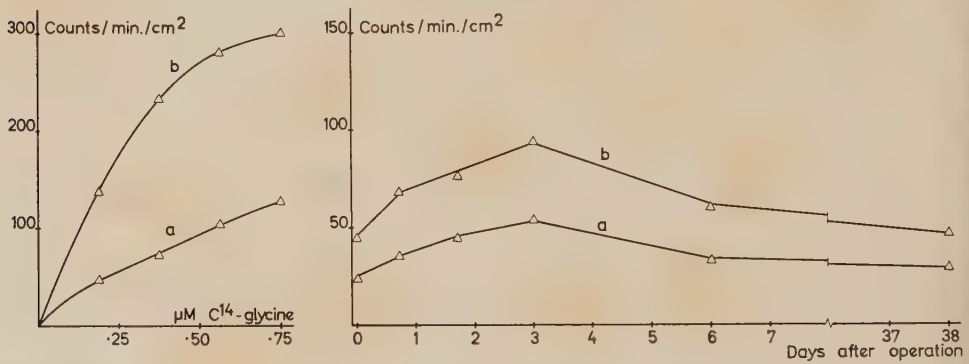


Fig. 5 (*left*).—Incorporation of C^{14}-glycine into glutathione in mitochondria-free rat liver homogenates prepared from (*a*) normal liver, (*b*) regenerating liver (40 hours). Effect of the concentration of C^{14}-glycine (μM/ml). After the incubation 20 mg of glutathione was added as a carrier. Fig. 6 (*right*).—Incorporation of C^{14}-glycine into glutathione in mitochondria-free rat liver homogenates prepared at different periods after partial hepatectomy. Incubation for (*a*) 5 min., (*b*) 10 min. After the incubations 20 mg of carrier-glutathione was added to each sample.

suggested that the activation of the glutathione formation in our material culminated slightly later than the activation of the protein metabolism.

The incorporation of glycine into glutathione takes place under the influence of a soluble enzyme [2]. It seemed possible that also the increased rate of incorporation of labeled amino acids into proteins might be due to an activation of soluble factors, i.e. the enzymes concerned with the formation of activated amino acid intermediates [5, 6]. The following experiments were carried out with the purpose to estimate the extent of activation in the soluble and in the microsomal components of the liver cytoplasm.

Mitochondria-free homogenates of standard concentration were prepared at the same time from normal and from regenerating (40 hours) livers. From both kinds of homogenates microsome and cell fluid fractions were prepared by the method described in the experimental section. In amino acid incorporation experiments microsomes from the normal as well as from the regenerating livers were combined with equal samples of cell fluid from both kinds of livers (Table I). The experiments showed that the activity of the amino acid activating enzymes of the cell fluid increased at regeneration and that this increased activity could be utilized by the system to promote a higher isotope incorporation into proteins, especially in the combinations with microsomes from regenerating liver. The activation of the microsomal ma-

TABLE I

Microsome (mic) and cell fluid (sup) fractions isolated under standard conditions from (N) normal and (R) regenerating (40 hours) rat liver. Incorporation of C^{14}-L-leucine and C^{14}-glycine into the proteins of systems composed of the microsome and cell fluid preparations in different combinations. C.p.m. per cm².

	C^{14}-L-leucine		C^{14}-glycine	
	N_{sup}	R_{sup}	N_{sup}	R_{sup}
N_{mic}	347	443	50	53
R_{mic}	550	852	82	106

terial, however, had a considerably higher influence on the isotope incorporation in these mixed systems than the activation of the soluble enzymes (Table I). It will be shown in a subsequent report that this activation is correlated with an increased content of ribonucleic acid in the microsomal material from regenerating livers.

SUMMARY

The incorporation into proteins of C^{14}-L-leucine and C^{14}-glycine was studied in mitochondria-free rat liver homogenates at different periods after partial hepatectomy. As a response to the hepatectomy the enzymatic systems concerned with the incorporation of these labeled amino acids into proteins became activated. The activity maximum was reached about 40 hours after the operations. At that period the activity of the homogenates were 2.5–3 times higher than in control homogenates from non-operated animals. The increased activity involved the amino acid activating enzymes in the cell fluid as well as the microsomal systems. However, the effects of the microsomal activation on the amino acid incorporation were more pronounced.

An increased rate of incorporation of C^{14}-glycine into glutathione was observed in the homogenates from regenerating liver.

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A MODIFIED SPENCER MICROTOME FOR THIN SECTIONING

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The Spencer 820 rotary microtome modified in the manner of Pease and Baker [13] or that of Newman, Borysko, and Swerdlow [12] was one of the first instruments used successfully in the cutting of single thin sections for electron microscopy. In order to obtain serial thin sections it has subsequently been found necessary to float the sections as they are cut, on the surface of a liquid. This has been accomplished by the use of a trough, which has been attached to the knife and filled to the cutting edge with fluid [6]. Certain difficulties, however, are encountered when a reciprocating instrument such as the Spencer microtome is used. The specimen will, as it passes close to the knife on the return stroke, draw with it the liquid in the trough and the section just cut. Furthermore, in the microtome modification of Neumann *et al.* the specimen, being continuously advanced by thermal expansion, will scrape across the knife on the return stroke. This results in damage to the block surface as well as to the knife edge.

Consequently, new microtomes for thin sectioning have been designed so that the specimen and the knife come in contact on the cutting stroke only [1, 4, 5, 7, 8, 9, 14, 15, 16, 17]. Two modifications of standard reciprocating microtomes have been described in which such contact between specimen and knife on the return stroke is avoided. In one the knife is moved back from the cutting plane [2]; in the other the specimen is displaced laterally to the knife [18].

The purpose of this paper is to describe the mechanics and operation of a Spencer 820 rotary microtome equipped for thermal advance, which has been further modified to achieve the desired by-passing of the knife by retraction of the specimen on the upstroke.

The standard microtome operates by means of a feed mechanism, which is linked to the drive mechanism in such a way that the object clamp is automatically advanced between cutting strokes. Forward motion is produced when the feed pawl engages the ratchet wheel behind the ride-over plate and

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turns the wheel counter-clockwise.[1] As a result the feed screw nut tip is moved along the inclined plane, thus advancing the object clamp. As cutting proceeds the ratchet wheel is moved in one direction only, e.g. counter-clockwise.

In the modified instrument the feed mechanism and the drive mechanism interact in such a way that the object clamp, which holds the thermal advance unit, is automatically retracted on the return stroke and brought back to its original position at the beginning of the cutting stroke. Translated into the dynamics of the feed mechanism this means that the ratchet wheel is made to move in two directions, clockwise for retraction and counter-clockwise for repositioning, traveling an equal distance in either direction.

Description of Retraction Modification

In order to obtain automatic retraction several changes have been made which are illustrated in Figs. 1 and 2. The linkage between the pawl arm and the cam on the flywheel shaft is removed. The pin holding the feed pawl on the arm is replaced with a longer one, which has been turned down at the outer end and has had a $\frac{3}{8}''$ O.D. ball bearing roller pressed on. This pin extends out to just clear the flywheel. A $\frac{1}{8}''$ thick cam of approximately 180 degrees plus a fall off on each end is mounted on the inner face of the flywheel so as to allow the ball bearing to ride on the inside edge of the cam on the return stroke.

The feed pawl is made to engage the ratchet wheel ahead of the ride-over plate, which is now used as a stop plate, by removing the two rollers. The spring between the pawl and the pawl arm is replaced by a clamp with a knurled head lock screw so that the pawl can be locked into the wheel at

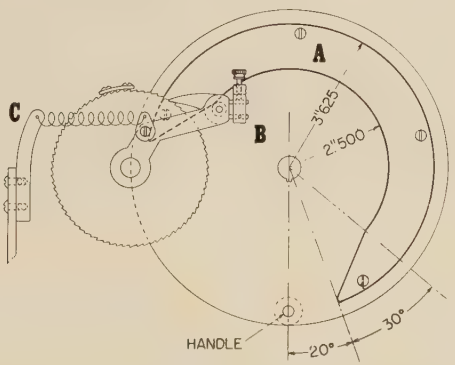


Fig. 1.—Drawing of modified feed mechanism. *A*, cam; *B*, pawl clamp; *C*, arm for retraction spring.

[1] In this report the terms counter-clockwise and clockwise refer to the direction of rotation when the ratchet wheel is viewed from the inside of the microtome, as in Fig. 2.

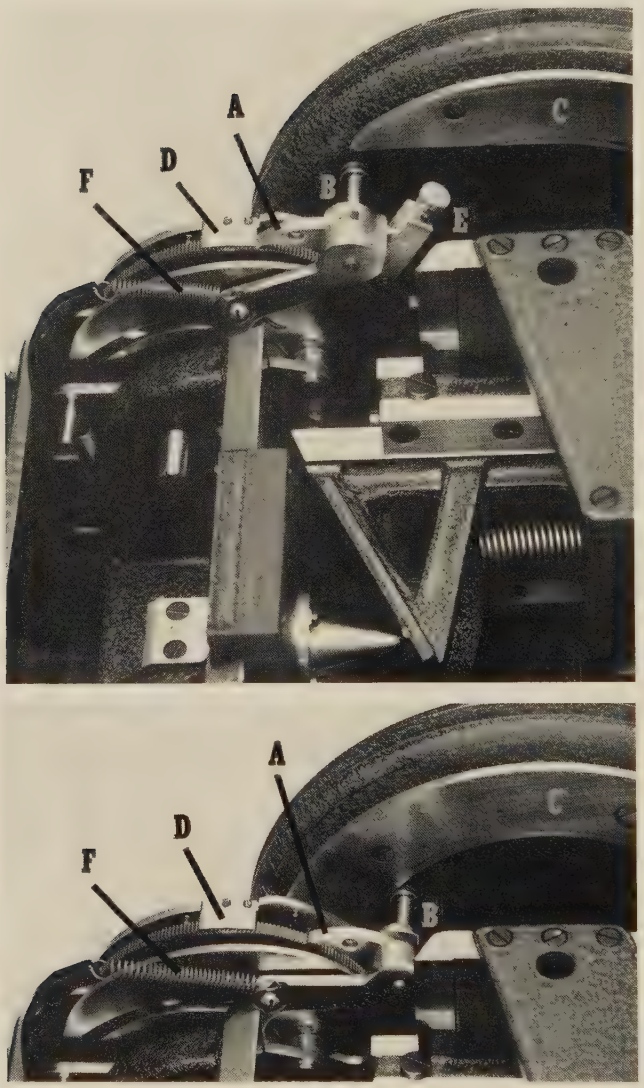


Fig. 2.—Position of feed pawl and ratchet wheel during cutting stroke (top) and during return stroke (bottom). *A*, pawl; *B*, roller pin; *C*, cam; *D*, stop-plate; *E*, pawl clamp; *F*, return spring.

any position. The clamp is fastened to the pawl pin with a set screw and has a slot to accommodate the pawl arm pin. This slot allows the pawl to be raised clear of the wheel when the feed handle is used to advance or retract the specimen. An arm is fastened to the inside back of the microtome case to hold a spring which is attached to the pawl arm at the other end.

The square headed friction screw at the base of the ratchet wheel is re-

moved to permit free passage of the cam, and a slot is cut in the cover of the case to provide clearance for the cam roller pin.

At the beginning of and during the cutting stroke (Fig. 2, top) the pawl (*A*), which is locked into the ratchet wheel, is held against the stop plate (*D*) by the spring (*F*). Retraction takes place at the end of the down stroke when the cam (*C*) on the flywheel engages the roller pin (*B*) and moves the pawl and wheel away from the plate. The object clamp remains in this retracted position (Fig. 2, bottom) as it passes the knife on the upstroke. At the top of the upstroke the roller pin is disengaged from the cam, and the spring returns the pawl and wheel to the stop plate. The object clamp is thus brought back to its original forward position at the beginning of the next cutting cycle.

The amount of retraction can be adjusted by rotating the feed buttom at the back of the microtome which controls the position of the stop plate and pawl in relation to the fixed flywheel cam. In Fig. 2 the plate is turned as far back as possible for a cam of this design. The maximum clearance is 13 microns as measured by the number of teeth the pawl has moved the ratchet wheel clockwise (Fig. 2, bottom).

Microtome Set-up

The modified feed mechanism is used only to accomplish by-passing of the knife, and actual advance of the block is produced through thermal expansion of the electrically heated specimen holder [3]. The voltage input is controlled with a variac, and the temperature changes are registered on a galvanometer, which is connected with the back of the specimen holder through a thermocouple (Fig. 3).

The microtome is run by a Bodine 60 r.p.m. type NSY-12R motor which is mounted independently of the microtome support. The ratio between the motor pulley and the microtome flywheel is 1:5, making the speed of the flywheel 12 r.p.m. The belt is made from $\frac{3}{16}''$ thick round leather belting. The motor is controlled by a toggle switch and by a momentary contact switch. The modified microtome may be operated by hand, but sections of a more uniform thickness have been obtained with the motor driven instrument.

Operation of Instrument

The knife is brought up to the specimen by moving the knife holder assembly forward, or the specimen is advanced to the knife by turning the feed handle. In order to do the latter the pawl clamp is loosened and the

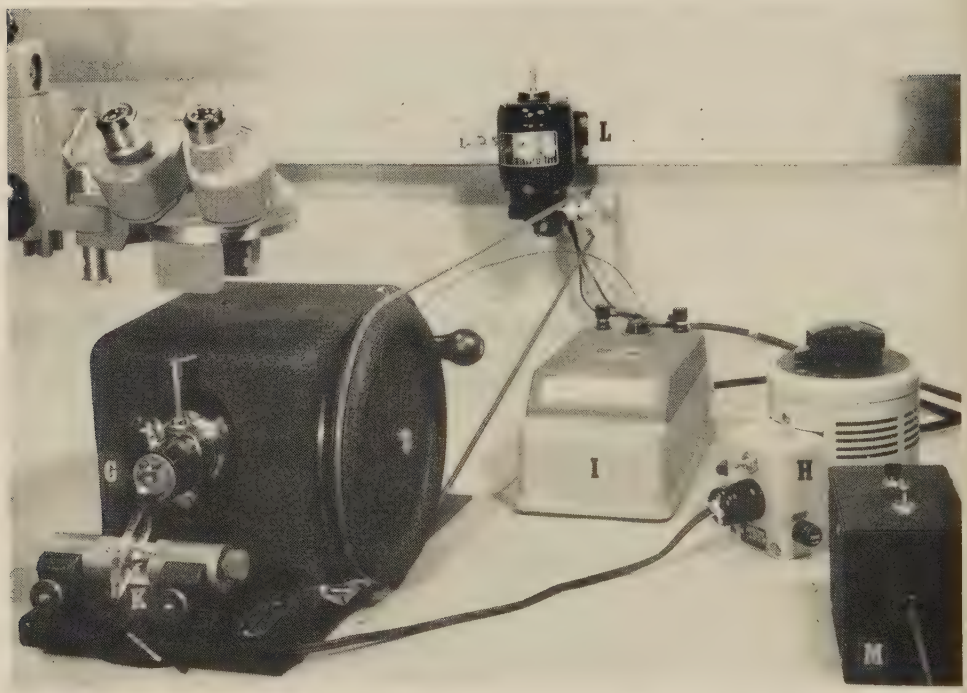


Fig. 3.—Microtome set-up. *G*, thermal advance specimen holder; *H*, variac; *I*, galvanometer; *K*, knife-holder; *L*, motor; *M*, motor switch box.

pawl raised free of the ratchet wheel. After the specimen and the block have been placed in near contact by either method the current to the specimen holder is turned on, and the pawl and ratchet wheel are temporarily used as a fine advancing mechanism. The pawl, which at the beginning of a cutting stroke is held against the stop plate, is raised free of the wheel and clamped down in a new position one or more teeth back of the plate. The spring returns the pawl to the plate and moves the wheel counter-clockwise with it. The specimen is thus advanced as many microns as the number of teeth the pawl was set back. The microtome is run for one full cycle using the momentary contact switch, and this procedure is repeated until the entire face of the block is being cut. At this point the pawl is left locked and the motor is turned on permanently. The amount of current passed through the holder is adjusted so that a section is obtained on each downstroke, and so that the sections do not show any interference colors. Further adjustments in current may be made as cutting proceeds if either of these criteria is not fulfilled.

Cutting Technique

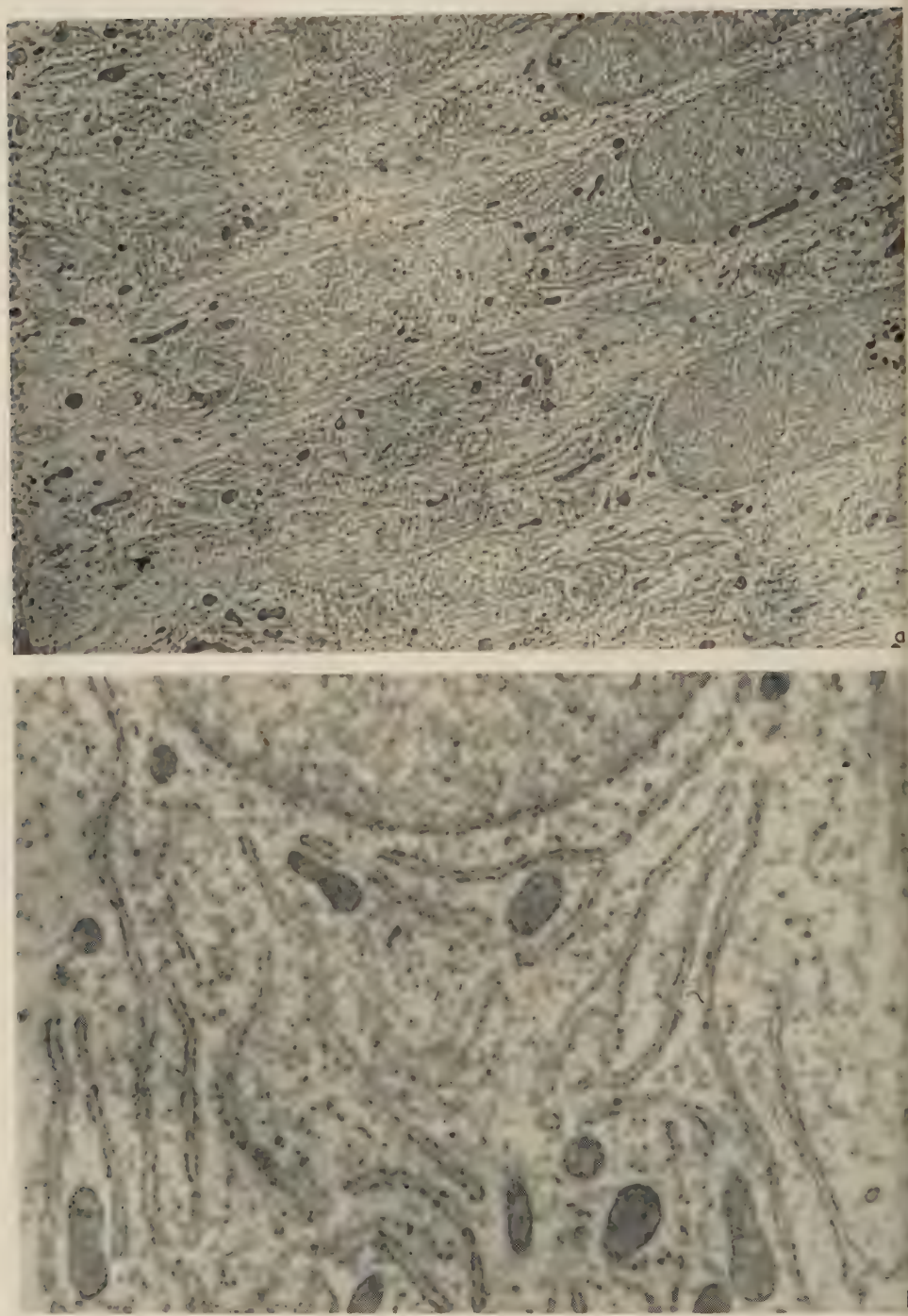
In this laboratory glass knives are used routinely [10] and are prepared daily. The knives are inspected under a reflection type microscope at 600 times magnification, and at best only a 1–2 mm region of the edge is perfect enough for cutting. The location of the good part of the edge relative to the left side of the knife is measured with a stage micrometer and recorded. A piece of black adhesive tape is next applied to form the trough and sealed to the knife with dental base-plate wax.

The methacrylate embedded specimen [12] is trimmed to a cube with its cutting surface not more than $\frac{1}{2}$ mm $\times$ $\frac{1}{2}$ mm and placed in the microtome chuck. The knife is set in the holder, and the good part of the cutting edge is relocated under the stereoscopic microscope either by use of a micrometer ocular or simply by measuring with a ruler. The knife angle is fixed at approximately 7 degrees. The cube is oriented with its front face parallel to the good part of the edge. After the knife and the specimen have been brought into contact in the manner previously described, the trough is filled with enough distilled water to yield a slightly convex surface. As sufficiently long ribbons appear on the water surface they are carefully pushed away from the knife edge with a single hair of a fine brush. They are lifted from the trough on the surface of a drop of water contained in a wire loop, 3 mm in diameter, and transferred to a distilled water surface [11].

The container with the floating sections is heated to 40°C for one hour and then allowed to cool to room temperature. The sections thus flattened are next transferred with the loop to a clean water surface for final washing. For mounting, the sections are picked up again with the wire loop, and an electron microscope screen, previously covered with a carbon substrate, is touched to the ribbon or dropped on top of it in the wire loop. The water is drained off the screen with filter paper. Single sections as well as ribbons may be handled in this way and they are easily placed at or near the center of the screen.

RESULTS

Three microtomes modified as described above have been constantly in operation in this laboratory for more than 8 months. A variety of tissues ranging from decalcified dentin to normal and virus infected soft tissues have been cut successfully. Fig. 4, *a* and *b* are electron micrographs of typical sections of the odontoblasts from the growing zone of a mouse incisor.



SUMMARY

A modification of the Spencer 820 rotary microtome to permit cutting of ribbons of thin sections suitable for electron microscopy is described. The feed mechanism has been changed in such a way that the specimen is automatically retracted to bypass the knife on the return stroke and brought back into position at the beginning of the cutting stroke.

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Fig. 4, *a* and *b*.—Odontoblasts from the growing zone of mouse incisor. Fixed in a mixture containing 1 per cent osmic acid and 1 per cent potassium dichromate, buffered at pH 7.2. Magnifications: *a*, 4100; *b*, 19,000.

CELLULAR LIPOPROTEINS

I. THE INSOLUBLE LIPOPROTEIN OF WHOLE LIVER CELLS[1]

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Bensley and Hoerr [3] proposed the name "ellipsin" for the residual structural substance left after extracting liver cells with weakly alkaline salt solution. They described the microscopic appearance of the extracted cells and reported that the structural substance "dissolved" in strongly alkaline solution, that it precipitated when the solution was made acid, and that it contained 30 per cent "fatty substances". Several later authors have reported the preparation, from cells or tissues, of a substance that "dissolved" in alkali and precipitated upon the addition of acid without, however, noting its lipid content or structural nature. Their work has been reviewed briefly by Wang *et al.* [13]. Recently, the lipid content, i.e. lipoprotein nature, and also the structural nature of such an alkali-dispersable substance have been recognized again, and it has been studied further [6, 7, 8, 12, 13].

It has now been found that this cellular lipoprotein is dispersed, but not actually dissolved, in alkaline solution. It has not been possible to dissolve it in any reagent which will leave it chemically intact. Attempts to dissolve it in solutions of varying pH, with or without the aid of detergents, chelating agents, urea, hexametaphosphate, or other reagents, have been unsuccessful. When the cellular lipoprotein is suspended, by means of a Potter homogenizer, in buffered salt solutions, the degree of dispersion increases slightly with increasing pH, until a pH of approximately 7 is reached. Above that point the increase in dispersion with increasing pH is considerable, but the lipoprotein tends to decompose. The effect of increasing the pH is almost the same

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in either the presence or absence of each of the various reagents mentioned above.

Cognizant of the highly insoluble nature of the "ellipsin" type cellular lipoprotein, we have explored the possibility of preparing it as a residue by the complete removal of the soluble cell constituents. Previous work [7] has given some indication that the insoluble lipoproteins of the various parts of the cell are approximately alike in chemical composition, although in the course of that work they were subjected to a high pH before analysis. If the insoluble lipoproteins of various parts of the cell are, in fact, approximately alike in chemical composition, and a given type of cell contains no considerable amount of any other insoluble, sedimentable substance, then the insoluble lipoprotein residue could be expected to have approximately the same chemical composition throughout. In order to test the chemical homogeneity of the residue we have used a method consisting of centrifugal fractionation, described below. The rationale for this method is explained in the discussion.

It seems to have been tacitly assumed by most workers that the extraction of cells with reagents such as salt solutions leaves a residue too complex to be the object of fruitful investigation. The results reported here indicate that such a residue, properly prepared, contains essentially only the insoluble lipoprotein of the cell.

METHODS

Preparation of cells.—Calf liver was frozen rapidly soon after the animal was killed and kept frozen until used. It was then cut into pieces, partly thawed at room temperature and forced through a 10-mesh monel screen by placing the screen over a mortar and macerating the tissue with a porcelain pestle. Approximately 50 g of the mash were mixed, by hand stirring, with 100 ml of cold (5°C) Solution I (0.16 *M* potassium chloride, containing 186 mg of iodoacetic acid per liter, as an enzyme inhibitor, and 5 g of sodium citrate per liter, to prevent clotting of any blood present). The mixture was then strained consecutively through one, four and eight layers of cheese cloth, the material being forced by squeezing slightly during the one and four-layer strainings. Microscopic examination of the suspension shows no connective tissue strands. There are present whole cells, clumps of cells, some broken cells and a few erythrocytes. *In all subsequent operations the material was kept between 3° and 5°C.*

Removal of the cellular constituents soluble in salt solutions.—The following was accepted as a standard method of removing the soluble constituents, after many less desirable methods were tried: The cell preparation was centrifuged at 48,000 *g* for 30 minutes, the supernatant discarded and the residue mixed in a glass Potter homogenizer for 15 minutes with 50 ml of Solution I. The suspension was placed in a 100 ml cylinder, the volume brought to 100 ml with Solution I, and the contents mixed by inversion. It was then centrifuged at 48,000 *g* for 30 minutes and the residue mixed

in the homogenizer for 15 minutes with 50 ml of Solution II (1 *M* potassium chloride, containing 186 mg of iodoacetic acid and 10 g of sodium citrate per liter, adjusted to pH 4.7 by the addition of concentrated hydrochloric acid). The suspension was placed in a 100 ml cylinder and the volume brought to 100 ml with Solution II. The contents were mixed by inversion and centrifuged at 48,000 *g* for 30 minutes. The mixing of the residue with Solution II and the centrifugation were repeated as described until the supernatant failed to give a test for protein or nucleic acid. The protein test was made by adding 1 part of 10 per cent trichloracetic acid to 2 parts of the supernatant. Orcinol was used to test for RNA and diphenylamine to test for DNA; 6 or 7 extractions with Solution II were required. Free lipid rose to the top of the tubes during the first few extractions.

Fractionation.—The residue from the above extractions was mixed once more with Solution II and centrifuged at approximately 80 *g* for 10 minutes. The residue was designated Fraction I and the supernatant centrifuged at approximately 1100 *g* for 10 minutes. The residue was designated Fraction 2 and the supernatant centrifuged at approximately 48,000 *g* for 30 minutes. This residue was designated Fraction 3. Each of the three fractions was mixed by hand stirring with 50 ml of Solution I, centrifuged at approximately 48,000 *g* and the residue lyophilized.

Exposure of the lipoprotein to various adverse conditions.—During development of the method of preparation described, the lipoprotein residue was prepared by similar methods which, however, involved exposure to various conditions causing decomposition. Among these were the following deviations from the method finally accepted. (*a*) The temperature of the preparation was allowed to increase to approximately 20°C during each centrifugation while it was being prepared. (*b*) After extraction with the salt solutions as described, the lipoprotein was washed with water three times by mixing in the Potter homogenizer and centrifuged at 48,000 *g*. (*c*) The Waring blender was used instead of the Potter homogenizer in the preparation of the lipoprotein. The blender causes the formation of numerous air bubbles, thus exposing the suspended particles of lipoprotein to a gas-liquid interface.

Chemical analysis.—For all analyses the lyophilized samples were dried to constant weight in a vacuum oven at 50°C.

Crude lipid was determined as follows: Approximately 100 mg of the lipoprotein was placed in a flask with 75 ml of a 2:1 chloroform–methanol mixture and heated in a water bath at 60°C for 2 hours. The mixture was transferred quantitatively to a medium porosity fritted-glass crucible, previously dried to constant weight in a vacuum oven at 50°C. After filtration, the crucible was dried to constant weight as before. The crude lipid was calculated as the difference.

Nitrogen was determined by a micro-Kjeldahl method [10]. Phosphorus was determined by the method of Allen [1]. Hexosamine was determined by the Boas method [4].

RESULTS

Table I shows the results of crude lipid, phosphorus, nitrogen and hexosamine analysis of lipoprotein fractions prepared by the standard method. The results were compiled from analysis of the three fractions from each

TABLE I

Comparison of the lipid, phosphorus, nitrogen and hexosamine content of the lipoprotein fractions.

	Content of fractions Mean % ± S.E.M.			Comparison of fractions *p* Values		
	Fraction 1 (F1)	Fraction 2 (F2)	Fraction 3 (F3)	F1 – F2	F1 – F3	F2 – F3
Crude lipid	45 ±0.82	44 ±0.75	44 ±0.44	*	*	*
Phosphorus	1.08±0.025	1.07±0.027	1.07±0.020	*	*	*
Nitrogen	8.2 ±0.149	8.6 ±0.099	7.9 ±0.064	**	*	**
Hexosamine	0.23±0.016	0.23±0.010	0.24±0.009	*	*	*

*p value greater than 0.05,
**p value less than 0.05.

TABLE II

Effect of adverse conditions on the homogeneity of the lipoprotein.

Fraction	Approximate centrifugal force × *g*	Nitrogen %
	(a) Effect of heat	
1	80	10.2
2	1,100	9.3
3	20,000	8.5
	(b) Effect of washing with water	
1	800	11.7
2	2,900	11.3
3	17,000	11.6
4*		5.0*
	(c) Effect of exposure to a gas–liquid interface	
1	300	10.4
2	2,200	10.2
3	14,000	3.8

* See text.

of six experiments. The fractions did not differ significantly in crude lipid, phosphorus or hexosamine content, but Fraction 2 has a higher nitrogen content than Fractions 1 and 3.

Table II shows the results of the experiments in which the lipoprotein was decomposed by exposure to adverse conditions. In the experiment in which

the lipoprotein became warm (Table II, *a*) lipid rose to the top of the centrifuge tubes at each high-speed centrifugation throughout the experiment. In the experiment using the standard method, in which the temperature was below 5°C throughout, lipid came to the top of the centrifuge tubes only during the first two or three centrifugations.

In the experiment in which the lipoprotein was washed with water (Table II, *b*) the following results were obtained: The wash water became turbid. The addition of 1 *M* acetic acid to this turbid wash fluid caused a precipitate to form, which, upon centrifugation, had a gray-tan color and a flaky appearance. When rolled in the finger, however, it became dark brown and felt somewhat greasy. Its nitrogen content is shown in Table II (Fraction 4 of *b*).

DISCUSSION

During the course of previous investigations, in which the insoluble lipoprotein of the nucleus was prepared by dispersing it in alkaline solution, its chemical complexity was evident. The protein part of the lipoprotein of rat liver nuclei was found, by two-dimensional paper chromatography, to contain predominately glycine, alanine, valine, leucine (and isoleucine), serine, aspartic acid and glutamic acid [11]. At least 17 amino acids were present (tryptophan would have been destroyed by the acid hydrolysis). Amino acid chromatograms of hydrolysates of the lipoprotein of calf liver nuclei were almost the same as those of rat liver nuclei, the same amino acids predominating [2, 5]. Paper chromatography of hydrolysates of the crude lipid fraction of the lipoprotein of calf liver nuclei showed the presence of choline, ethanolamine, serine and a hexose, while hydrolysates of the whole lipoprotein yielded a hexosamine [2]. Engbring and Laskowoski [9] reported that carbohydrate was present in similar preparations, which had also been exposed to strong alkali.

After the alkali-dispersable lipoprotein was found to be insoluble in alkali, an extended period of investigation was devoted to attempts to dissolve it in other reagents mentioned earlier, without success. It was found, however, that it is decomposed easily by suspension in water or very dilute salt solution, by suspension in alkaline solution, by exposure, while in suspension, to a gas–liquid interface, or by other adverse conditions.

When the insoluble cellular lipoprotein is prepared, as in the present investigation, by mechanically breaking the cells and extracting the cellular constituents soluble in salt solution, the resulting residue can probably be thought of, with some accuracy, as pieces of various structures, some broken

and some in their original size. The pieces of broken structures would be analogous to pieces of a mosaic structure having a repeating pattern, the mosaic having been made of several kinds of material. If the unit size of the repeating pattern of such a mosaic were small enough so that each of the pieces would contain at least several units, then analysis would show the small pieces to have the same composition as the larger pieces.

The fractionation method, described above, was devised to test the homogeneity of the insoluble cellular residue. Each fraction collected by this method necessarily differs somewhat from the other fractions in the size distribution of the particles sedimented, although there is considerable overlapping of the size ranges. The method is based on the assumption that, if there were present in considerable quantity a second insoluble, sedimentable component, quite different from the first in chemical composition, the probability is small that the two components would be present in the fractions in proportional quantities. If the components were not present in the fractions in proportional quantities, variation in composition would be found among the fractions and the amount of the variation would depend on the difference in composition of the components and the degree of disproportional distribution. If the fractions are found not to differ significantly in chemical composition, this may be taken to indicate that the material is homogeneous, i.e., that the particles of various sizes are similar in chemical composition, insofar as the analysis was performed. If there were present two or more components almost alike in composition, such as two cellular lipoproteins, then the difference in chemical composition of the fractions would be small in spite of a disproportionate distribution of the components.

In the present investigation, the lack of significant differences between fractions of the cellular residue in their crude lipid, total phosphorus and total hexosamine content, points strongly toward the presence of only one component or the presence of two or more components similar in composition. The residue seems, then, to consist essentially of one or more complex lipoproteins. The small difference between Fraction 2 and each of the other fractions in nitrogen content can hardly be interpreted as indicating the presence of a large quantity of a component other than lipoprotein, in view of the lack of significant differences between fractions in crude lipid, phosphorus and hexosamine content.

The presence of hexosamine in the cellular residue might suggest that a mucopolysaccharide or mucoprotein is present as an additional component. The proportional distribution of the hexosamine among the fractions points, however, to its being an integral part of the complex. It should be pointed out

that it has been a common practice to subject substances prepared from tissues or cells to extraction by lipid solvents to remove "contaminating" lipid, or, conversely, to extract the tissue or cells with lipid solvents to obtain the total lipid as starting material for lipid studies. Therefore, there has been little opportunity to find a naturally occurring complex containing lipid, protein and carbohydrate, especially if that complex will not dissolve in any reagent while intact.

Any similarity in properties, especially in gross chemical composition, exhibited by cellular lipoproteins as a class of substances, is at present a point of interest, as is usual during the period when a class of substances is little known, e.g. nucleic acids ca. 1905. It seems reasonable to expect, however, that it will later become possible to demonstrate specific differences in chemical composition, and in other properties among various cellular lipoproteins, where such differences exist.

SUMMARY

The insoluble lipoprotein of whole calf liver cells was prepared as a residue by breaking the cells and extracting exhaustively with molar salt solutions. This lipoprotein residue was divided by centrifugation into three fractions, which were found to have the same crude lipid, phosphorus and hexosamine content and almost the same nitrogen content. It is concluded that the insoluble cellular residue is composed of lipoprotein essentially homogeneous in composition.

We are indebted to Dr. Addison Gulick for his interest and encouragement during the earlier part of this work and to the Armour Research Laboratories, Chicago, Illinois for generously supplying animal tissue through Armour and Company, Kansas City, Kansas.

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CELLULAR LIPOPROTEINS

II. A "GHOST CELL" PREPARATION[1]

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The concept that many cellular membranes or other cellular structures are composed of protein and lipid in intimate association has long been known. In the investigation reported here, the structural substance (lipoprotein) of liver cells is obtained for chemical analysis in such a way that it is made up of "ghost cells", that is, the gross aspects of the structure of the cells remain intact for microscopic observation. Further investigation will be required to determine how much of the fine structure is preserved. This "ghost cell" preparation is somewhat like that of Bensley and Hoerr [1], which they called "ellipsin", and differs from that previously reported from this laboratory [4] in that the latter had the structure of the cells mechanically broken.

Recent morphological studies indicate that certain membranes of the cell, formerly regarded as separate structures, are actually parts of a continuous structure, e.g., the nuclear envelope and the cytoplasmic membranes (ergastoplasm or endoplastic reticulum) [3, 5] or the latter and the cell membrane [3]. In view of this evidence, it is perhaps not surprising to find that the lipoprotein preparation already mentioned [4] (with the cell structure broken) is essentially homogeneous in chemical composition. The "ghost cell" lipoprotein preparation is shown below to have the same lipid content and nearly the same nitrogen content as the "broken cell" preparation.

METHODS

Preparation of cells.—Calf liver was removed from the animal soon after killing and forced through a 10-mesh monel screen by placing the screen over a mortar and macerating the tissue with a porcelain pestle. (In some experiments the liver was frozen rapidly soon after the animal was killed and kept frozen until used; the results were the same as those obtained with unfrozen liver.) Approximately 150 g of the

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mash were mixed, by hand stirring, with 1200 to 1500 ml of cold (5°C) 0.14 *M* sodium chloride solution. This and all solutions used subsequently contained merthiolate (1:10,000). The mixture was strained through two layers of cheese cloth, which removed the connective tissue and the larger aggregates of cells. In all subsequent operations the material was kept between 3° and 5°C.

Removal of cell constituents soluble in salt solutions. –The crude cell preparation was centrifuged for one half hour at approximately 120 *g* (just fast enough to cause sedimentation of the whole cells, but not fast enough to cause them to be packed tightly together). All subsequent centrifugations used in this procedure were done at the same speed and for the same time. Most of the cytoplasmic fragments and nuclei of broken cells remained suspended and were discarded with the supernatant. The remainder were removed by subsequent extractions. The residue was then suspended in approximately 500 ml of 0.4 *M* sodium chloride and stirred continuously for 10 to 12 hours with an electric stirrer, at approximately 350 rpm. It was important not to stir too rapidly and to center the stirring rod so that the movement was not turbulent enough to cause the cells to be beaten together and broken. All subsequent stirring was done in the same way. The suspension was again centrifuged. The residue was resuspended in about 500 ml of cold 0.7 sodium chloride and stirred for 3 to 4 hours. The suspension was centrifuged and the supernatant discarded.

The residue from the 0.7 *M* sodium chloride extraction was then extracted with 1.0 *M* sodium chloride alternately at about pH 7 and pH 4. For the pH 7 extraction, the material was suspended in 1 l of Solution I (1 *M* sodium chloride to which a saturated solution of veronal was added to bring the pH to 7). It was then stirred for 3 to 4 hours and centrifuged. For the pH 4 extraction, the residue was placed in 1 l of Solution II (1 *M* sodium chloride solution, containing 1 g potassium acid phthalate per liter, adjusted to pH 3 by adding 1 *N* hydrochloric acid). When the residue from pH 7 extraction was suspended in this buffer, the pH of the resulting suspension was between 3.7 and 4.1. This was stirred for 3 to 4 hours and centrifuged. The extractions at pH 7 and pH 4 were done alternately until no more soluble protein or nucleic acid remained. This required about seven extractions at pH 7 and seven at pH 4. The residue was tested for completeness of extraction as follows: Approximately 1 g of the residue was placed in 50 ml of Solution I. A similar portion was placed in 50 ml of Solution II. Each of these was homogenized in a Waring blendor for 1 hour at full speed and then centrifuged for 1 hour at 20,000 *g*. The supernatant fluid from each was tested for protein by the biuret and Millon tests, for RNA by the orcinol reaction and for DNA by the diphenylamine reaction.

When the extraction of the soluble proteins and nucleic acids was complete, the residue was washed with water[1] three times, briefly, by hand stirring and centrifuging. The residue consisted almost entirely of whole "ghost cells", the quantity of cell debris being negligible. A small part of the residue was used for microscopic observation and the remainder lyophilized for chemical analysis.

Microscopic examination.—The "ghost cells" and unextracted cells were suspended in 0.14 *M* sodium chloride and observed by means of a phase contrast microscope. The "ghost cells" and unextracted cells were also embedded in Carbowax (to avoid contact with lipid solvents), sectioned and stained for microscopic observation.

[1] The time of exposure to water was too brief to cause decomposition.

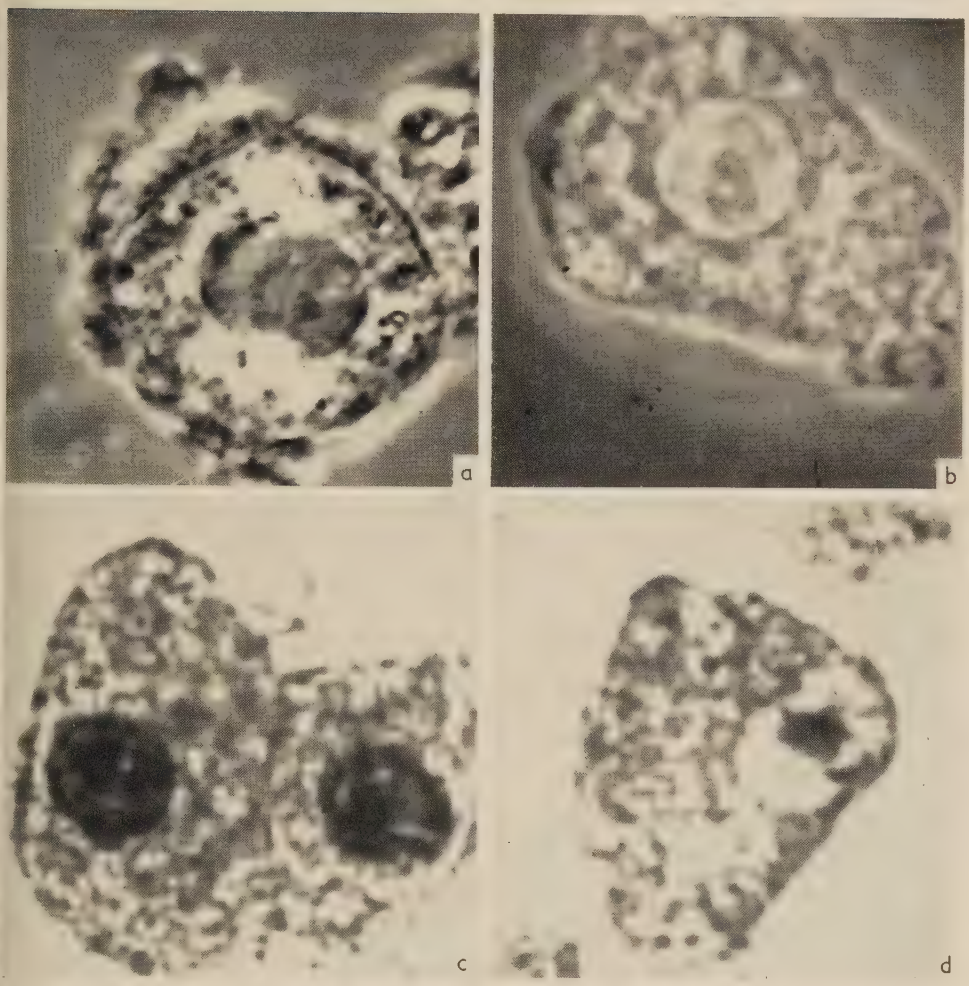

Fig. 1.—Unextracted and "ghost" calf liver cells. (*a*) Unextracted, phase contrast; (*b*) "ghost", phase contrast; (*c*) unextracted, sectioned, hemotoxylin-eosin stain; (*d*) "ghost", sectioned, hemotoxylin-eosin stain.

Chemical analysis.—For all analyses, the lyophilized samples were further dried to constant weight in a vacuum oven at 37°C. Crude lipid was determined by extraction in a Goldfisch extraction apparatus. Approximately 200 mg samples were placed in alundum thimbles, previously dried to constant weight in a vacuum oven at 37°C, and extracted with a chloroform–methanol mixture (2:1) for 40 hours. The residue was dried as before and the crude lipid calculated as the difference. Nitrogen was determined by a micro-Kjeldahl method [2].

RESULTS

Microscopic appearance of the "ghost cells". Photomicrographs of extracted "ghost cells" and unextracted cells are shown in Fig. 1. In the former the gross structure of the cell remains. The nucleus has approximately the same size and shape as before extraction but appears to be almost empty. There is present a nucleolus-like body, and sometimes filamentous structures are present in the nucleus. The cytoplasm is less dense than that of the unextracted cells and somewhat more granular. It is notably different from the nucleus in having no large empty spaces.

Chemical composition. The results of crude lipid and nitrogen analysis of five "ghost cell" lipoprotein preparations (each prepared in a different experiment) are shown in Table I, and those results are compared with the results of crude lipid and nitrogen analysis of "broken cell" lipoprotein preparations [4]. Crude lipid and nitrogen determinations, made on a lipoprotein residue prepared by using for extraction the solutions described previously [4], but stirring and centrifuging as described above, gave: Crude lipid, 45 per cent; nitrogen, 8.2 per cent. For those two determinations, the methods described previously [4] were used.

Ultraviolet light absorption measurements, made on hydrolysates of the

TABLE I

Comparison of the "ghost cell" lipoprotein with the "broken cell" lipoprotein (4).

Mean % ± S.E.M.

	"Ghost cell" Lipoprotein (GCL)	"Broken cell" Lipoprotein		
		Fraction 1 (F1)	Fraction 2 (F2)	Fraction 3 (F3)
Crude lipid	44 ±0.70	45 ±0.82	44 ±0.75	44 ±0.44
Nitrogen	8.1±0.126	8.2±0.149	8.6±0.099	7.9±0.064

p Values

(**p* value greater than 0.05; ***p* value less than 0.05.)

	F1 – GCL	F2 – GCL	F3 – GCL
Crude lipid	*	*	*
Nitrogen	*	**	*

"ghost cell" lipoprotein with a Beckmann DU spectrophotometer, showed no indication of the presence of purines or pyrimidines, indicating the absence of nucleic acid.

DISCUSSION

A "broken cell" lipoprotein preparation was previously reported [4] to be composed essentially of one component or of two or more components very similar in chemical composition. Since the "ghost cell" preparation has the same lipid content and nearly the same nitrogen content as the "broken cell" preparation, these two preparations seem to be composed essentially of the same cellular lipoprotein or lipoproteins. The close similarity in the chemical composition of these two preparations, in spite of the long period of time required for the preparation of the "ghost cell" lipoprotein, indicates considerable stability, in contrast to the ease with which the lipoprotein is decomposed by certain influences, such as exposure to water.

The outer cell boundary and the boundary of the nuclear space usually retain, in the "ghost cell", approximately their original size and shape, although the nuclear space is almost empty. This indicates considerable rigidity in the cytoplasmic structure.

The method of attack used here seems to offer a means for further investigation of the chemical nature of the structural substance of the cell or its parts, thus relating the chemical nature of the substance to its morphological role. Preliminary investigation has indicated that the fine structure of at least certain types of cells can be observed in the "ghost cells" by means of electron microscopy.

SUMMARY

The insoluble lipoprotein of calf liver cells was prepared as a "ghost cell" residue by extracting exhaustively with salt solutions without breaking the cells. The "ghost cell" lipoprotein had the same crude lipid content and almost the same nitrogen content as the "broken cell" lipoprotein previously reported. Photomicrographs of unextracted and "ghost cells" are shown.

We are indebted to Dr. Addison Gulick for his interest and encouragement during the earlier part of this work and to the Armour Research Laboratories, Chicago, Illinois for generously supplying animal tissues through Armour and Company, Kansas City, Kansas.

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A PHOTOELECTRIC RECORDING SET FOR PULSATION CURVES OF HEART MUSCLE CULTURES *IN VITRO*[1]

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A HIGH percentage of heart muscle explants continue *in vitro* to effect rhythmic pulsations, a fact well known to those acquainted with tissue culture work. This contractile activity depends not only on extrinsic factors (temperature, composition of the culture medium, and so on), but also on intrinsic ones, such as the vitality, the age, and the topographical origin of the chosen fragment, its relation to the impulse-conducting system [3]. Consequently, there are some differences in the behavior of the various explants; nevertheless, considering a given preparation, we can very well appreciate the different comportment of the muscle contractions before and after an experimental influence. Hence, we are able to demonstrate the action of a cardiotonic drug producing a direct effect upon the pulsating myocardium which is, of course, subjected to neither nervous nor humoral control in tissue culture.

We are working with hanging drop cultures of heart muscle from chicken and mouse embryos, maintained in a medium consisting of blood plasma and only very little homologous embryonic extract. The explanted fragments must be small enough to permit the exchange of nutritive substances and metabolites by diffusion. The coverslip bearing the culture is mounted on a special chamber allowing perfusion with an appropriate nutritive solution of defined composition. A two-way cock enables us to change the perfusing liquid, i.e. either to replace it by the same fluid nutrient medium now containing, however, a suitable concentration of the drug whose action we want to examine, or to replace it by quite another relevant solution in which we are interested.

If we intend to study *in vitro*, under physiological and experimental conditions, the pulsation activity of different parts of the myocardium (and, possibly, even of isolated muscle fibres) of healthy and altered hearts, we need information about the amplitude and the frequency of the contractions. We can determine the latter without difficulty by simple counting; it is, how-

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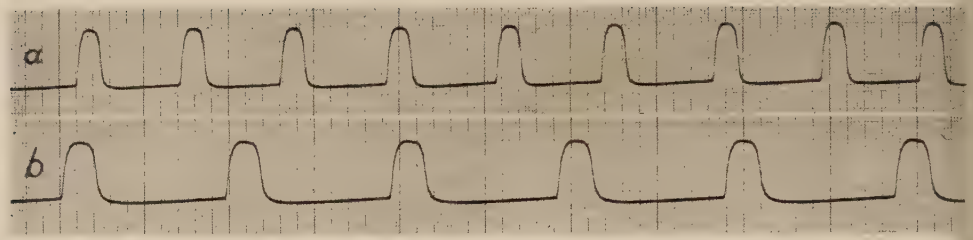

Fig. 1.—Two examples of pulsation curves obtained from the same heart muscle explant of a 6-day-old chicken embryo: (*a*) the culture having been maintained at a temperature of 39°C; (*b*) after a one-hour sojourn at 33°C.

ever, much more difficult to evaluate the size of the amplitudes, very often of special interest to us. Starting from the assumption [1], proven by preliminary trials, that the light quantity passing through the beating myocardial fragment varies during contraction and relaxation, we thought that it should be possible to register contraction curves by the photoelectric method. We therefore developed a recording set which we would like to describe in the following paragraphs (see also [2]). This installation enables us to complete the information obtained by photomicrographic motion pictures with results acquired by quantitative appreciation of the contraction curves. Of course, these curves, though finally inscribed by an electrocardiograph, have nothing to do with real electrocardiograms (due to the action current) such as have been established, also from myocardial cultures, by O. Olivo [4] and other workers.

The microscope is placed in an incubator, the temperature of which (38–40°C) is regulated thermostatically and inscribed with a thermograph for control, because the contractile activity is very susceptible to temperature variations (Fig. 1). We can add phase contrast equipment, but generally we have good results even without it, using an ordinary objective of 10 times magnification (Wild Fluotar) and a photo eyepiece of 10 times magnification. As light source, we use a low voltage filament lamp fed by a storage battery. Since we intend to register the light variations due to the muscle pulsations, it goes without saying that we must work with an absolutely stable current, i.e. direct current, as the oscillations of the alternating current would also be recorded. Because too high an intensity of light injures the beating myocardial tissue, it is very important to intercalate a heat-absorbing filter and to reduce the level of illumination as much as the sensitivity of the photoelectric recording set and, if we ever take graphic pictures at the same time, that of the photographic film will permit.

There is another essential point. The light fluctuations caused by the muscle pulsations are extremely small in comparison with the total brightness of the entire field of view. Therefore, for the photoelectric only, not for the microcinematographic recording, we cut out a slit with a diaphragm

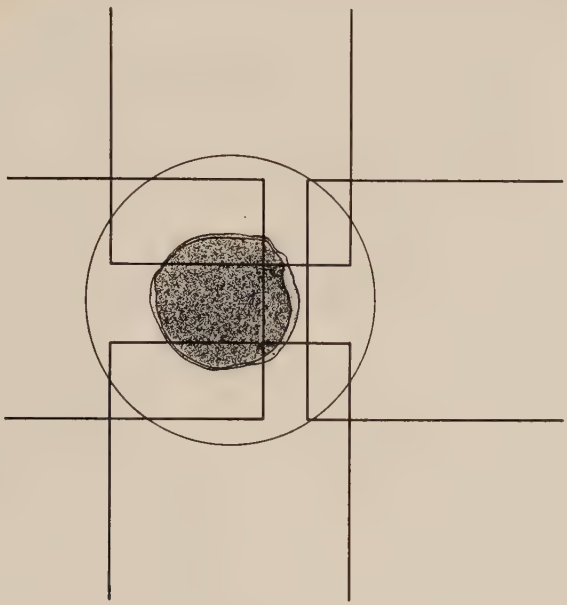

Fig. 2.—Schema of the microscopic field of view (circle) with the pulsating culture and the adjustable diaphragm composed of four polarizing plates which cut out a rectangular slit.

(Fig. 2). In this slit, the light variations are now relatively great, and much more so when the cleft has been adjusted at the place of the greatest amplitude of the contractions. The fact that, by the interposition of a diaphragm, the absolute light energy reaching the photoelectric cell is considerably diminished is rather irrelevant, because it can easily be compensated by a higher amplification of the electric impulses.

After having presented these fundamental considerations, we shall briefly describe the principal parts of our apparatus. The tube of the microscope is prolonged by an eking-piece containing two prisms as shown schematically in Fig. 3. The first of these deviates some per cent of the light into a viewer which permits focusing and observation of the preparation; in Fig. 3, this viewer (V_1) is directed to the right and placed immediately above the incubator (I). The second prism lets half the remaining light pass to the motion picture camera (Paillard H 16 with objective Yvar 75 millimeters) and reflects the other half into the annexed installation containing the photomultiplier (PM). This installation (see also Fig. 4), one of the main parts

of the recording set, is sketched in Fig. 3 on the left side of the eking-piece. Moreover, we see that a third prism is inserted before the light rays attain the photomultiplier, again diverting some per cent of the light into a second viewer (V_2). Between the second and the third prism, we find the diaphragm

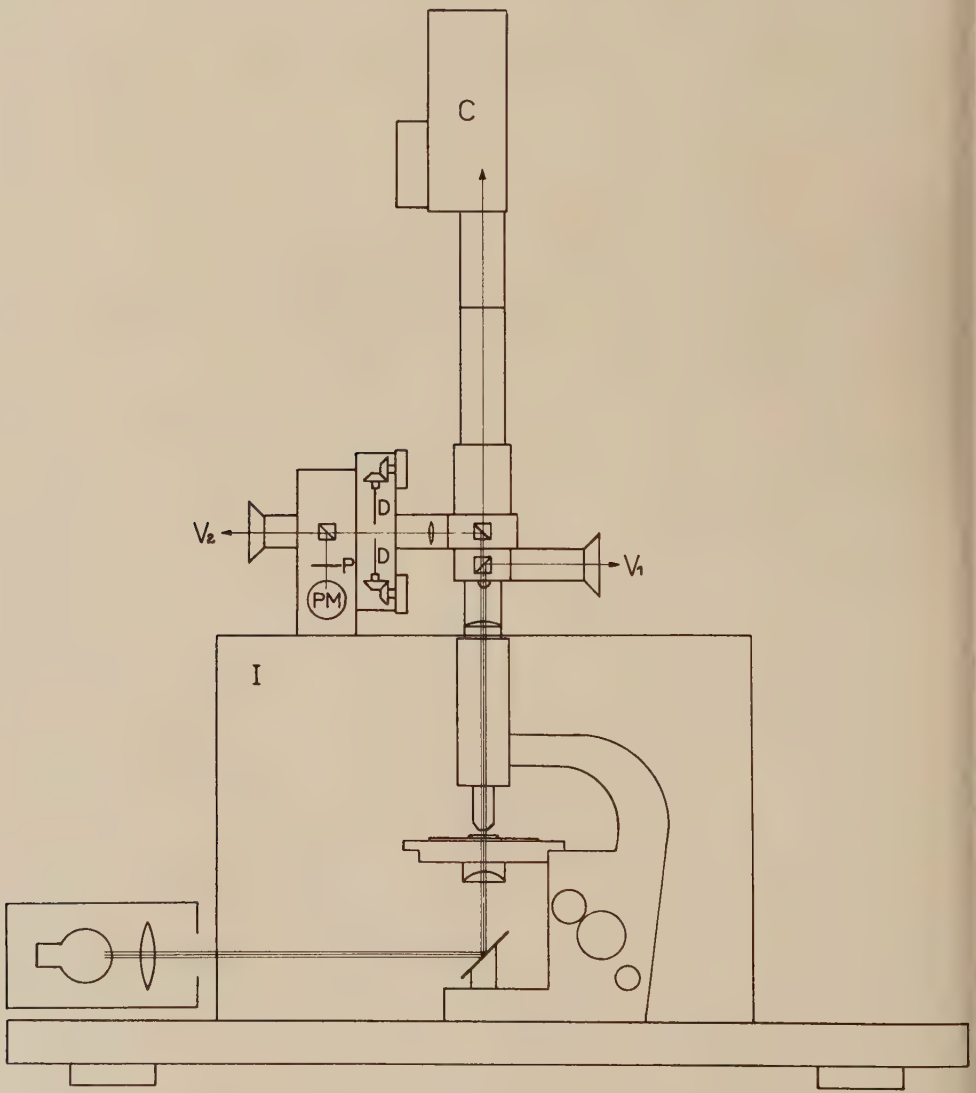

Fig. 3.—Schema of the optical part of the recording set (for details see text). *C*, motion picture camera; *D*, polarizing diaphragm; *I*, incubator, including the microscope; *P*, second polarizing filter; *PM*, photomultiplier; V_1, first viewer; V_2, second viewer; ◺ ◺ ◿, prisms.

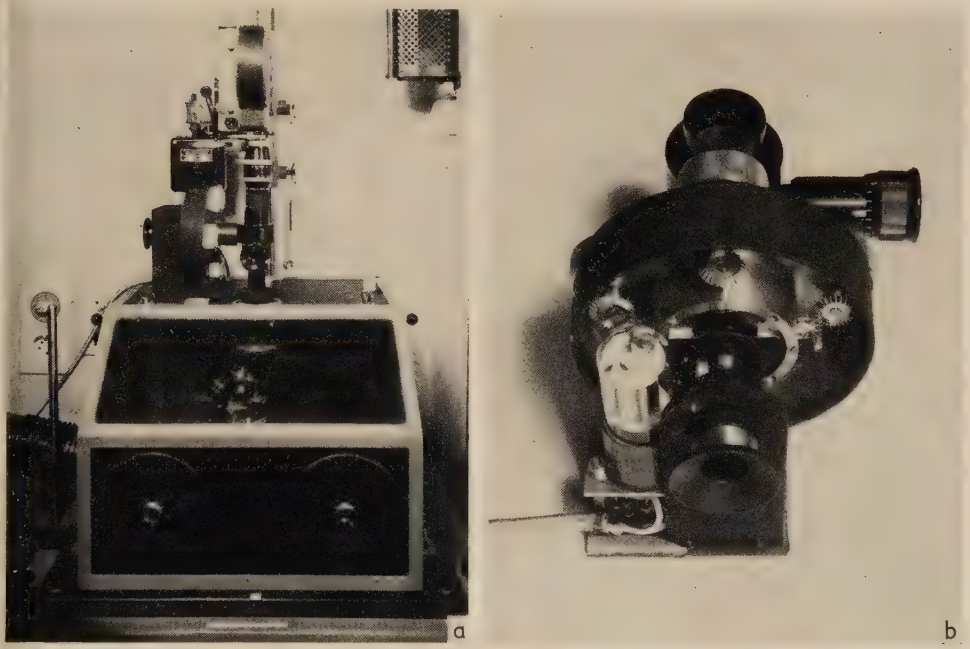

Fig. 4*a* and *b*.—Photographs: (*a*) showing a general view of the optical part (schematically represented in Fig. 3); (*b*) a detail of the (opened) installation containing the photomultiplier (electronic tube), the second viewer, the third prism, and the diaphragm adjustable by four screws, three of which are visible; above, pointing to the right, the first viewer.

already mentioned (*D*) and in front of it a converging lens focusing the image of the preparation at the level of the diaphragm. This is composed of four plates which are individually adjustable by means of four screws (Fig. 4*b*). The plates consist of a transparent but polarizing material (polaroids). Thus, the light beams passing through the slit as well as those going through the diaphragm can reach the second viewer in so far as they have not been diverted by the third prism; that is, in the second viewer we see not only the slit but the whole preparation, and therefore we have the possibility of controlling the manipulation of the diaphragm and of adjusting the slit where it is most convenient. Between the third prism and the photomultiplier, however, we put in another polaroid which, if its axis is oriented perpendicularly with regard to the axis of the diaphragm polaroids, extinguishes the polarized light and allows only the passage of the light not yet polarized, i.e. the light coming through the slit (Fig. 2), the intensity of which changes with contraction and relaxation of the beating heart fragment.

The photoelectric cell used by us (photomultiplier, visible in Fig. 4*b*) is a very sensitive electronic tube RCA 1 P 21 in connection with an apparatus for its high tension electric feeding (Fig. 5). This apparatus also gives us the possibility of augmenting or diminishing the current feed and, therewith,

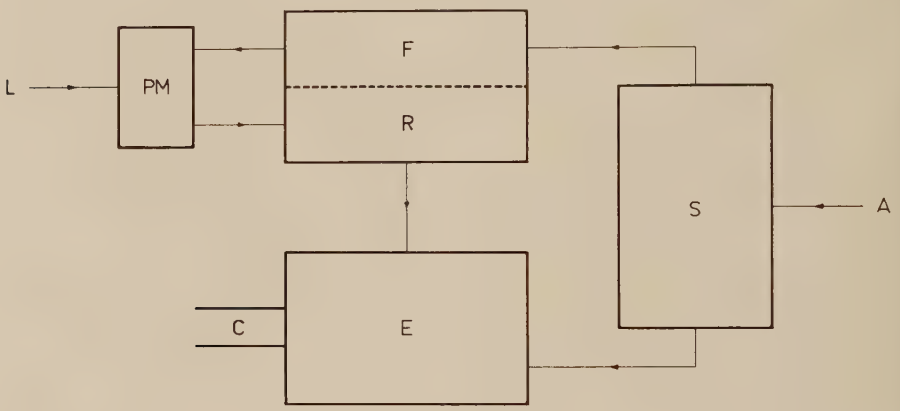

Fig. 5.—Schema of the electric part of the recording set (for details see text). *A*, ordinary alternating current; *C*, recorded pulsation curve; *E*, electrocardiograph; *F*, high tension current feeder for the photomultiplier; *L*, light beams coming through the slit (Fig. 2); *PM*, photomultiplier; *R*, regulator of the electric impulses emitted by *PM*; *S*, current stabilizer.

of adjusting the sensitivity of the electronic tube; furthermore, it permits the adapting (reducing) of the current emitted by the photomultiplier. These electric impulses, varying with the light fluctuations due to the myocardial pulsations, are finally recorded by an electrocardiograph equipped with a sensitivity regulator and a stabilizer for the current feed. Naturally, the above-mentioned installation, which furnishes the current for the electronic tube, must also get its supply of electricity by way of a stabilizer. Since we are obliged to work with very high amplification, one of the great problems in the construction of the electric part of the recording set—which is, in detail, somewhat more complicated than has been outlined here—was the elimination or, at least, minimization of accidental disturbances. As for the electrocardiograph, we take a type (cardiostat super of Siemens) which inscribes directly on a paper band moving forward at a speed of 25 millimeters per second. Hence, we can see immediately the result of the experiment in the behavior of the curves (Fig. 1). These, moreover, are an objective document of the results which can be later evaluated quantitatively.

SUMMARY

The fact that the light quantity passing through hanging drop cultures of beating heart muscle fragments varies during the pulsations, offers the possibility of recording contraction curves by the photoelectric method. These curves, which are informative not only with regard to the frequency but also to the amplitude of the pulsations, show us immediately, when registered by a directly inscribing electrocardiograph, the result of an experimental influence and can be evaluated quantitatively.

Such a photoelectric recording set, as it has been developed by the author, is described in this paper.

We wish to thank the "Fonds national suisse de la recherche scientifique" for its support which made the realization of this work possible. We would also like to express our gratitude to Doctor B. Vittoz of the "Institut de physique technique et mécanique" of the University of Lausanne for his most valuable technical assistance and for the development of the construction plans. We are also indebted to the optical manufacturer Wild Ltd. of Heerbrugg (Switzerland) for the optical calculations.

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STUDIES ON THE CONVERSION OF FOLIC ACID TO CITROVORUM FACTOR BY DEVELOPING CHICK EMBRYO[1]

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The morphological changes occurring in the chick embryo during development have been extensively studied. In recent years, an attempt has been made to bring to embryology the various chemical techniques which have proved to be of value in other areas of biology and to explain the changes taking place during development on a chemical basis [18–20]. With this in mind studies have been conducted in this laboratory on the different chemical changes occurring in the chick embryo during development [10, 11, 24, 25].

The presence in chick liver of an enzyme system converting pteroyl glutamic acid (PGA) to citrovorum factor (CF) has been reported [6, 7, 8, 21]. The importance of this enzyme system has become evident in recent years in that reports have appeared indicating an involvement of folic acid derivatives in the interconversion of serine and glycine [1, 2, 16], in the *de novo* synthesis of the methyl group of methionine [9] and in the biosynthesis of purines [4, 12]. The present report concerns the study of the effect of age of the embryo on PGA to CF conversion by chick embryo homogenate and chick embryo liver homogenate. Preliminary observations have also been made on the effect of X-radiation on the conversion.

EXPERIMENTAL

Factors affecting conversion of PGA to CF by chick embryo homogenate and chick embryo liver homogenate.—Eggs from straight run (New Hampshire males × Single Comb White Leghorn females) crossbred chicks were obtained from the Poultry Plant of Texas A. and M. College. The eggs were incubated in a commercial incubator at 37.5°C. After various periods of incubation, eggs were taken out and the embryos were tested for their capacity to convert PGA to CF. The number required for each study depended on the stage of development. In the later stages one or less sufficed,

[1] This work was supported by a grant from the American Cancer Society. Folic acid and leucovorin were generously supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. The authors are indebted to Dr. R. J. Shalek for the calibration of radiation dosage.

TABLE I

Cofactor requirements for the conversion of folic acid (PGA) to citrovorum factor (CF) by chick embryos.

Omission*	Addition*	CF synthesized
		μg/g
None		25.0
PGA		5.9
Serine		3.9
Homocysteine		4.6
DPN		12.8
ATP		18.8
None	Mg++	19.8

* The reaction mixture contained 100 μg PGA, 10 mg serine, 5 mg homocysteine, 2 mg DPN, 4 mg ATP and 3 ml of homogenate (equivalent to 25 mg dry weight) of 2.5-day-old chick embryo prepared in 0.08 *M* sodium potassium phosphate buffer, pH 6.6; total volume 4 ml. Magnesium chloride was tested in 10 mg quantity. Flasks were incubated under N_2 for 3 hrs at 37°C and were later autoclaved at 120°C for 30 min.

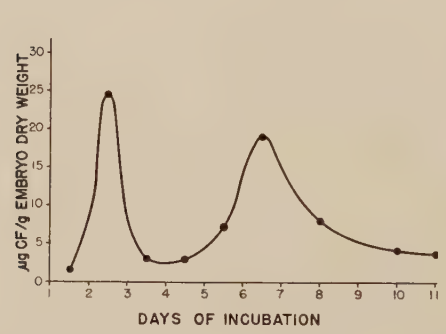
but in the early stages many embryos were pooled in order to have sufficient material for a single experiment. Conversion studies were carried out on whole embryos and on isolated embryo livers. The latter organ was chosen because in the adult animal the liver has the highest capacity to convert PGA to CF.[1] Whole chick embryos or chick embryo livers were homogenized in 0.08 *M* sodium potassium phosphate buffer, pH 6.6 using a Potter–Elvehjem glass homogenizer. An aliquot of the whole embryo homogenate (equivalent to 25–30 mg dry weight) or of the embryo liver homogenate (equivalent to 80–100 mg dry weight) was used for PGA to CF conversion studies. The effect of serine, homocysteine, DPN, ATP and Mg^{++} on the conversion of PGA to CF by chick embryo homogenate was studied (Table I). The measurements on the conversion of PGA to CF by chick embryo homogenate or chick embryo liver homogenate were made by determining the difference between the CF synthesized by the embryo homogenate or the embryo liver homogenate in a complete system with PGA and the amount of CF present in a control experiment without PGA (Figs. 1 and 2). The samples were incubated at 37°C under nitrogen for 3 hrs. At the end of the incubation period the incubation mixtures were neutralized to pH 6.8 and autoclaved for 30 min. at 120°C. After cooling, the samples were homogenized in a Waring blendor, made up to volume, filtered and the filtrate was assayed. The CF content of the filtrates from whole embryos or from the embryo livers was determined by using the Bacto-CF assay medium (Difco Laboratories, Inc.). *Leuconostoc citrovorum* ATCC 8081[2] was used as the test organism and leucovorin (Lederle) was employed

[1] Doctor, V. M., Unpublished data.

[2] A report (Felton, E. A. and Niven, C. F., *J. Bacteriol.* **65**, 482 (1953)) concerning a taxonomic study on the culture *Leuconostoc citrovorum* A. T. C. C. 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae* as described earlier (Pederson, C. S., *Bacteriol. Revs.* **13**, 225 (1949)).

as the standard. The cultures were incubated for 48 hrs. at 37°C, and growth was measured by turbidimetric procedure. Since leucovorin has been reported to be half as active as the CF isolated from a horse liver [15], the microbiological assay values are divided by 2 in order to express the results in terms of the naturally occurring CF.

It was of interest to determine if the CF synthesized by chick embryo homogenate or the chick embryo liver homogenate from added PGA was identical with synthetic CF (Leucovorin, Lederle). This was accomplished by analyzing the filtrate prepared



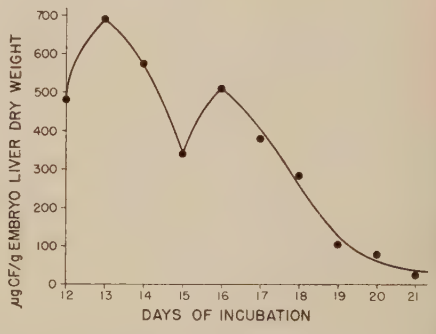


Fig. 1 (*left*).—Conversion of PGA to CF by chick embryo homogenate. The reaction mixture contained 100 μg PGA, 10 mg serine, 5 mg homocysteine, 2 mg DPN, 4 mg ATP and 3 ml of homogenate of chick embryo (equivalent to 25–30 mg dry weight); total volume 4 ml. Flasks were incubated under N_2 for 3 hrs at 37°C and were later autoclaved at 120°C for 30 min. Control incubations were simultaneously run with all the above components of the reaction mixture without PGA. The data on μg CF/g embryo homogenate represents the difference between CF synthesized by the embryo homogenate in a complete system with PGA and the CF present in the control experiment without added PGA.

Fig. 2 (*right*).—Conversion of PGA to CF by chick embryo liver homogenate. The reaction mixture contained 100 μg PGA, 10 mg serine, 5 mg homocysteine, 2 mg DPN, 4 mg ATP and 3 ml of homogenate of chick embryo liver (equivalent to 80–100 mg dry weight); total volume 4 ml. Flasks were incubated under N_2 for 3 hrs at 37°C and were later autoclaved at 120°C for 30 min. Control incubations were simultaneously run with all the above components of the reaction mixture without PGA. The data on μg CF/g embryo liver homogenate represents the difference between CF synthesized by the embryo liver homogenate in a complete system with PGA and the CF present in the control experiment without added PGA.

for CF assay using bioautographic procedures described by Broquist *et al.* [3]. The results of this study indicated that the compound synthesized from PGA by either chick embryo homogenate or the chick embryo liver homogenate moved on paper strips at the same rate as did synthetic CF (Leucovorin, Lederle).

Effect of X-radiation on the conversion of PGA to CF by chick embryo.—Chick embryos were removed from eggs and immediately suspended in ice cold physiological saline. One half of the embryos served as unirradiated controls. The other half was irradiated at 20°C, with a 200 KV X-ray source delivering 600 r/min. The irradiated embryos received 15 min. exposure and thus a total radiation of 9000 r. Immediately after irradiation the irradiated embryos and the unirradiated controls were homogenized in 0.08 *M* sodium potassium phosphate buffer, pH 6.6 with the Potter–Elvehjem glass homogenizer. The conversion of PGA to CF by the embryos

was studied as described above. The reaction mixture contained 3 ml homogenate of chick embryos (equivalent to 25–30 mg dry weight), 100 μg PGA, 10 mg serine, 5 mg homocysteine, 2 mg DPN, and 4 mg ATP in a total volume of 4 ml. Control incubations were also run with all the above components of the reaction mixture without PGA. Flasks were incubated under nitrogen for 3 hrs. at 37°C and were later autoclaved at 120°C for 30 min. Samples were cooled and assayed for CF content as described above.

RESULTS AND DISCUSSION

It is apparent from the results of Table I that the components required for the optimal conversion of PGA to CF by chick embryo homogenate include PGA, serine, homocysteine, DPN and ATP, while addition of Mg^{++} decreases the conversion. Magnesium chloride is also reported to inhibit the conversion of PGA to CF by chick liver homogenate [7].

Effect of age of the embryo on its capacity to convert PGA to CF is plotted in Fig. 1. It may be noted that at 4th and 10th days of incubation, there are depressions in the enzyme activity as measured by the decrease in the synthesis of CF. Also, there are two peaks of CF synthesis at 2.5 and 6.5 days of incubation. Determination of CF synthesis by chick embryo livers from 12th day through hatching is plotted in Fig. 2. The results indicate depressions of CF synthesis at 12th and 15th days of incubation and peaks at 13th and 16th days of incubation. It is interesting to note that on dry weight basis embryonic liver is 20–25 times more efficient in converting PGA to CF than the whole embryos (compare Figs. 1 and 2). The results also suggest a close relationship between the depressions observed in the conversion of PGA to CF by chick embryo homogenate at 4th and 10th days (Fig. 1) and by chick embryo liver homogenate at 12th and 15th days (Fig. 2) and the depressions observed by Schmalhausen [23] in the growth of whole embryos at 4th, 9th, 12th and 15th days during incubation. During the conversion of PGA to CF, the chick liver supernatant solutions are reported [5] to convert PGA to a compound which, upon heating, gives CF. N^{10}-Formyltetrahydropteroylglutamic acid has been reported to undergo a rearrangement upon autoclaving with the formation of CF [22]. Furthermore, both the precursor of CF and N^{10}-formyltetrahydropteroylglutamic acid are labile to oxygen and can yield CF under anaerobic conditions [14]. Thus a measure of CF synthesized by the chick liver in the present experiment may also be indicative of the amount of N^{10}-formyltetrahydropteroylglutamic acid accumulation during the incubation period. Since N^{10}-formyltetrahydropteroylglutamic acid is reported to be the active formyl donor for the biosynthesis of purines

Table II

Effect of X-radiation on the conversion of folic acid to citrovorum factor by chick embryo.

Age of the embryo (days)	CF synthesized*	
	Controls (μg/g)	Irradiated (μg/g)
2.5	27.5	29.3
6	12.7	10.8
7	16.0	17.2
8	10.8	11.8

* Immediately after irradiation (9000 r) the irradiated embryos and the unirradiated controls were homogenized in 0.08 *M* sodium potassium phosphate buffer, pH 6.6 using the Potter–Elvehjem glass homogenizer. The reaction mixture contained 100 μg PGA, 10 mg serine, 5 mg homocysteine, 2 mg DPN, 4 mg ATP and 3 ml homogenate of chick embryo (equivalent to 25—30 mg dry weight) prepared in 0.08 *M* sodium potassium phosphate buffer pH 6.6; total volume 4 ml. Flasks were incubated under N_2 for 3 hrs. at 37°C and were later autoclaved at 120°C for 30 min.

[13], an increase in the conversion of PGA to CF may also indicate an increased rate of utilization of N^{10}-formyltetrahydropteroylglutamic acid for purine synthesis.

Results on the effect of X-radiation on the conversion of PGA to CF by chick embryos are summarized in Table II. These data indicate that under experimental conditions described above, the conversion is not affected by X-radiation. The incorporation of formate-C^{14} into the DNA adenine, guanine and thymine by 14-day old chick embryos is reported to be 50 per cent lower in embryos irradiated with 400 r X-radiation than in the unirradiated controls [17].

SUMMARY

The components required for the optimal conversion of PGA to CF by chick embryo homogenate include PGA, serine, homocysteine, DPN and ATP. The chick embryos possess maximum capacity to convert PGA to CF at 2.5 and 6.5 days during the incubation period of 1.5 to 11 days. The conversion of PGA to CF by chick embryo liver homogenate is optimal at 13 and 16 days during 12 to 21 days of incubation. Under the experimental conditions described here, 9000 r X-radiation to 2.5, 6, 7, and 8 days old chick embryos does not alter their capacity to convert PGA to CF.

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THE PROTEOLYTIC ACTIVITY OF THE GASTROPOD EGG[1]

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The activity of three peptidases in the gastropod egg has been previously studied [1, 2] and in the present work evidence for two additional proteolytic enzymes, one peptidase and one proteinase, is presented. The characterization of these enzymes is based on their substrate specificity and pH optimum.

METHODS

The gastropod *Nassarius festivus* was kept in an aquarium of running sea water and eggs were collected from the aquarium soon after they had been deposited. The proteolytic activity was determined by homogenizing 5 eggs or embryos in 2 ml distilled water with subsequent incubation at 38°C with 7 microliters of 0.05 *M* leucineamide in veronal buffer. The degree of hydrolysis was determined by titrating the free carboxyl groups according to Weil's [4] micro-modification of the formol titration. Titrations were carried out in a medium of 40 per cent formaldehyde with 0.05 *N* NaOH using 0.1 per cent alcoholic phenolphthalein as an indicator. The activity was obtained by subtracting the titration value of a blank, i.e., a reaction vessel minus eggs, from the titration value of the substrate plus the homogenized eggs. All determinations were done in quadruplicate, and the activity has been arbitrarily expressed as microliters of 0.05 *N* NaOH per egg or embryo. A Scholander microburette, with a sensitivity of 0.15 ml, was used for the titrations.

RESULTS

In Fig. 1, the hydrolysis of leucineamide at various pH values is given, and it is clear that there are two optima, one at pH 4.6 and a second at pH 8.2. The activity at pH 4.6 corresponds to the pH range of the catheptic enzymes while that at 8.2 indicates peptidase activity. The only two animal enzymes known to hydrolyze leucineamide are cathepsin III at a pH of 4

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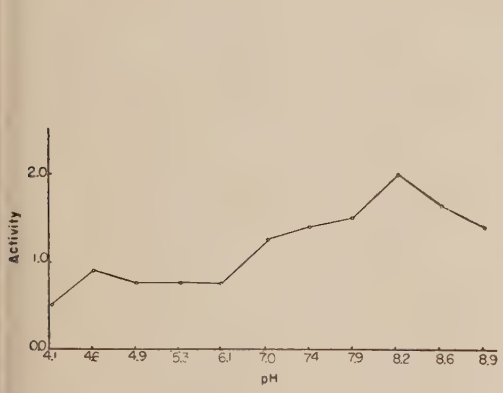
to 5 and leucine aminopeptidase around pH 8.0. The addition of 0.01 *M* cysteine as an activator for the catheptic enzymes failed to cause any increase in the hydrolysis of leucine amide at pH 4.6; similarly, 0.02 *M* ascorbic acid exhibited no activating effect at this pH. This fact does not necessarily indicate that a cathepsin is not involved as the determination of activity was carried out on an unpurified homogenate and the presence of an



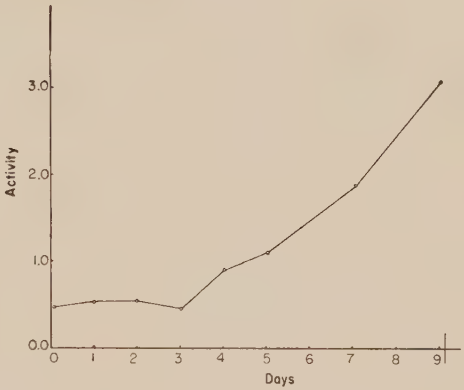


Fig. 1 (*left*).—Hydrolysis of leucineamide versus pH.
Fig. 2 (*right*).—Activity of cathepsin III during the course of normal development.

activator in the homogenate is not ruled out. Thus, from these data it is concluded that the gastropod egg has a proteolytic activity corresponding to that of cathepsin III and leucine aminopeptidase.

Whether these two activities are to be interpreted as being due to two distinct enzymes, i.e., two different proteins, cannot be decided on the basis of the available evidence. Gutmann and Fruton [3] working with crystalline chymotrypsin, were unable to separate an endopeptidase and aminopeptidase activity in a highly purified preparation; thus, the possibility exists that a single protein species may be responsible for these two enzymatic activities.

The activity of cathepsin III was determined throughout the course of development and it was found, as shown in Fig. 2, that an increase in activity occurred at about the fourth day of development (at 20°C). Following this initial rise in activity, a gradual increase continued until the seventh day of development at which time the veliger larva was fully differentiated. Thus, the pattern of catheptic activity during the development of the gastropod egg follows the same general course as was previously found [1, 2] for the activity of the peptidases studied.

SUMMARY

Two proteolytic enzymes, cathepsin III and leucine aminopeptidase, have been identified in the gastropod egg. The activity of cathepsin III was followed throughout the period of embryonic development.

The author wishes to express his most sincere appreciation to Professor Katsuma Dan and the Tokyo Metropolitan University for the many kind favors extended to him during his stay in Japan. Gratitude is also expressed to Professor I. Tomiyama and the Staff of the Misaki Marine Biological Station for their generous hospitality in making available the facilities of their station.

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Experimental Cell Research **13**, *125–131 (1957)*

PHOSPHOLIPID CONTENT IN NOVIKOFF HEPATOMA, REGENERATING LIVER AND IN LIVER OF FED AND FASTED NORMAL RATS

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The object of our investigation of the Novikoff hepatoma is the finding of therapeutically exploitable biochemical differences between this tumor and normal liver. Our previous studies have concentrated on the elucidation of glucose-6-phosphate utilization in the Novikoff hepatoma and the glucose-6-phosphate utilization pattern of this tumor was compared to that of normal resting and fast-growing liver [10–11]. To separate the nutritional effects on the transplanted tumor from the original biochemical changes in the neoplastic tissue, the effect of fasting on glucose-6-phosphate utilization was also explored.

Parallel with these carbohydrate metabolic studies, recently studies were started on carbohydrate metabolism of the Novikoff hepatoma. The present paper compares the phospholipid content of Novikoff hepatoma and of normal and regenerating liver. The effect of fasting on the phospholipid content of normal rat liver is also presented.

MATERIALS AND METHODS

Male, adult Wistar rats weighing 180–220 g were used in these experiments. For regenerating liver studies the animals were partially hepatectomized (66 per cent) under light ether anesthesia, according to the method of Higgins and Anderson [5]. Sham operations were performed at the same time on rats of the same weight. At periods of 1, 3, 8, and 22 days following the operation, four to eight partially hepatectomized and the same number of sham-operated rats were sacrificed. The Novikoff hepatomas were 7 or 8 days old when used. Purina Fox Chow and water were available *ad libitum* at all times except for the fasting animals which received only water *ad libitum* and were isolated in separate cages during the fasting period.

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[1] Senior Fellow of the Cancer Research Society.

[2] This project has been supported by a grant from the Cancer Research Society, Montreal, Canada. Grantee of the National Cancer Institute of Canada.

The animals were killed by a blow on the head and were decapitated and bled. The livers were quickly removed, blotted on filter paper, and minced with scissors. From the minced liver duplicate samples of 500 mg each were weighed and placed in a mixture of hot alcohol and ether. The tissue phospholipids were extracted following the procedure of Schmidt *et al.* [8]. The phosphorus of phospholipids was determined by the method of Fiske and Subbarow. The colorimeter readings were done at 450 mμ with Beckman Quartz Spectrophotometer, Model DU, in glass cells No. 2097. The micro-Kjeldahl procedure was employed for total nitrogen determinations. The number of nuclei per gram of fresh liver was counted by the technic of Price and Laird [7], as modified by Allard *et al.* [2]. The cellularity was expressed according to Ultman *et al.* [9], as the number of nuclei per unit wet weight of tissue.

The tissue phospholipid content was expressed as μ grams of phospholipid phosphorus per g wet weight, per mg nitrogen, and per average cell.

RESULTS

Comparison of phospholipid content of normal rat liver and Novikoff hepatoma.—The phospholipid content of normal liver and Novikoff hepatoma is shown in Table I. The liver of normal fed rat was found to contain 1364 μ grams of phospholipid phosphorus per gram wet weight and this number agrees with data published in the literature. This means 42.2 μ grams of phospholipid phosphorus per mg nitrogen or 6.3×10^{-6} μ grams of phospholipid phosphorus per average cell. The phospholipid content of the Novikoff hepatoma was markedly decreased to 44 and 73 per cent of the normal value when the phospholipid phosphorus was expressed per gram wet weight or per mg nitrogen respectively. The decrease of phospholipid in the Novikoff hepatoma was even more marked when it was expressed per average cell (32 per cent). This was due to the higher cellularity of the Novikoff hepatoma as compared to normal fed rat liver [10].

TABLE I

Comparison of phospholipid content of normal and neoplastic liver.

Tissue	Phospholipid P μg per g wet weight	Phospholipid P μg per mg nitrogen	Phospholipid P μg per cell $\times 10^{-6}$
Normal liver[a]	1364±257	42.2±4.9	6.3±0.9
Hepatoma[a] (Novikoff)	605±137	30.6±4.1	2.0±0.5
Change in per cent[b]	44	73	32

[a] Means and standard deviations of 10 animals are given.
[b] The values of normal liver are taken as 100 per cent.

Rate of restoration of total phospholipid content.—The rate of restoration of total liver constituents and total liver phospholipid content is shown in Figs. 1 and 2.

Rate of liver regeneration.—Fig. 1 shows the changes in liver weight, liver/body weight ratio, total liver nitrogen, and total number of liver cell nuclei over a 22-day period subsequent to partial hepatectomy. The varia-

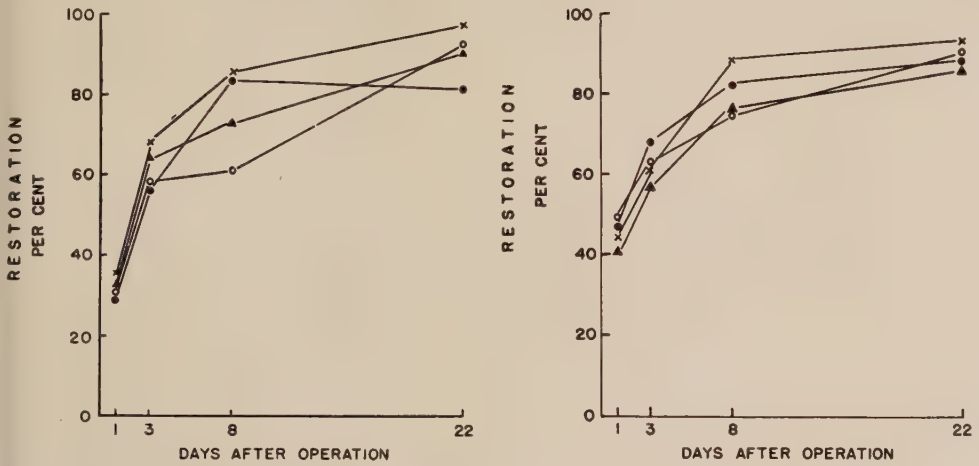

Fig. 1 (*left*).—Restoration of liver constituents after partial hepatectomy. The restoration of liver values is expressed in per cent, and the values of the sham-operated animals are taken as 100 per cent, for each interval. ●———● total number of liver cells; ○———○ total liver nitrogen; ×———× liver/body weight ratio; ▲———▲ liver weight.

Fig. 2 (*right*).—Restoration of total liver phospholipid content after partial hepatectomy. The restoration of the total liver phospholipid is expressed in per cent, and the values of the sham-operated animals are taken as 100 per cent for each time interval. ●———● total liver cell phospholipid content; ○———○ total liver phospholipid content per nitrogen; ×———× total liver phospholipid per body weight; ▲———▲ total liver phospholipid content (wet weight basis).

tions are expressed as per cent changes from the values of the sham-operated animals of the corresponding intervals. The values of the sham-operated animals of the corresponding intervals are taken as 100 per cent. The comparison of the values of the partially hepatectomized animals to the values of sham-operated animals was found necessary to exclude changes due to anesthesia, surgery, lower food intake, wound-healing, and the reaction of the organism to this stress situation [12].

The data show that there was an almost complete restoration of the liver weight, liver/body weight ratio, total liver nitrogen, and total number of liver cells during the 22-day period. The restoration of liver/body weight ratio was the most rapid and the restoration of the total liver nitrogen was the slowest.

TABLE II

Comparison of the phospholipid content of normal, neoplastic and regenerating liver in rat.

Tissue	Phospholipid P[a] μg per g wet weight	Phospholipid P[a] μg per mg nitrogen	Phospholipid P[a] μg per cell × 10^{-6}
Normal liver[b]	100	100	100
Hepatoma (Novikoff)[b]	44	73	32
Regenerating liver[c]			
1 day	123	144	148
3 days	123	105	114
8 days	105	104	85
22 days	93	99	101

[a] Values are expressed in per cent.
[b] The value of normal liver is taken as 100 per cent.
[c] The values of the sham-operated control animals of the same interval are taken as 100 per cent.

Rate of restoration of total liver phospholipid content after partial hepatectomy.—Fig. 2 shows the total liver phospholipid content as per cent changes when the values of the sham-operated animals of the corresponding intervals are taken as 100 per cent. The liver phospholipid contents for each of the investigated time intervals have been expressed on the basis of total liver weight, liver/body weight ratio, total number of liver cells, and total liver nitrogen. Fig. 2 shows that the total liver phospholipid is restored more rapidly at 1 day after partial hepatectomy than the other liver constituents examined (Fig. 1). After 1 day the restoration of total liver phospholipid content went nearly parallel with the restoration of other liver constituents. The liver phospholipid content was restored to nearly normal total liver values by the 8th day. At 22 days the total liver phospholipid content almost reached the corresponding values of sham-operated animals.

Comparison of the phospholipid content per unit in normal, regenerating liver, and in hepatoma.—In Table II are compared the phospholipid content of normal liver, hepatoma, and regenerating liver. The phospholipid content is given as μ grams of phosphorus per gram wet weight, per mg nitrogen, and per average cell. In this comparison the values are expressed in per cent. The values of normal liver are taken as 100 per cent. For the regenerating liver values the values of the sham-operated animals of the corresponding interval are taken as 100 per cent. Table II shows that at 1 day after partial hepatectomy there was a marked increase in the phospholipid content of the regenerating liver as expressed per unit. This increase is most marked when expressed per average cell. At 3 days there was an apparent increase

which was, however, insignificant when expressed per mg nitrogen or per average cell. The phospholipid content per unit was normal at 8 days and 22 days after partial hepatectomy.

The effect of fasting on liver phospholipid content.—Animals bearing fast-growing transplanted tumor may develop anorexia and do not eat although food is offered *ad libitum*. This fasting condition in itself may influence the

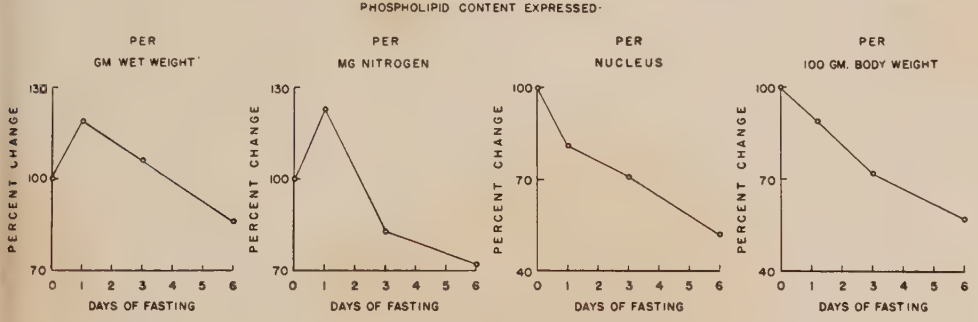

Fig. 3.—The effect of 6-day fasting on liver phospholipid content.

biochemical composition of tissues. To separate the effects of fasting from that of the neoplasia, the effect of fasting in normal animals has been explored. The effect of fasting on the liver phospholipid content of the normal rat is presented in Fig. 3. The phospholipid content was followed for 6 days. Fig. 3 clearly shows that the basis of expression of the phospholipid content is of great importance. When phospholipid content is expressed per wet weight or per nitrogen content, an apparent increase is noted at 1 day fasting. This is due to the shrinkage of the liver. To counteract the shrinkage of the liver and the loss of nitrogen, the phospholipid content is also expressed per average nucleus. Phospholipid content per nucleus shows a steady decrease during fasting. After 6 days fasting, the average liver cell contained only 50 per cent of the phospholipid content of the liver of a normal fed rat. When the phospholipid content is expressed per 100 g body weight a similar slope of decrease was noted.

DISCUSSION

The marked decrease in the phospholipid content of the Novikoff hepatoma is in line with reports on decreased phospholipid content of another type of rat hepatoma [4]. It is interesting to consider the decreased amount of phospholipid content in the neoplastic liver, since phospholipid synthesis is a primary function of all cells in the body. Phospholipids make up part of the cell structure, being found both in the cytoplasm and nucleus. Mitochon-

dria and microsomes are very rich in phospholipids. It was established that the rate of phospholipid synthesis by the liver cell is constant when related to a unit of tissue or nucleoprotein and is not influenced by the protein content of the diet, fat content of the liver, or degree of fibrosis [3].

The loss of phospholipid content from the neoplastic liver cell may reflect the tendency of the hepatoma cell to lose its specialized functions. The Novikoff hepatoma cell has largely lost its ability to store glycogen which is reflected in markedly decreased phosphoglucomutase activity [13]. The Novikoff hepatoma has also lost its ability ro release sugar, which is reflected in the absent glucose-6-phosphatase activity [11] in this tumor. Similar decreases were found in other specialized functions of the primary liver tumors caused by the feeding of the dye 4-dimethylaminoazobenzene [6].

Since a large part of the phospholipid content of the liver cell is concentrated in the mitochondria, it is important to consider the fact that the number of mitochondria is markedly decreased in the Novikoff hepatoma [1]. Preliminary studies on the comparison of the phospholipid content in the intracellular fractions of normal liver and Novikoff hepatoma showed that the most pronounced decrease (a loss of 77 per cent) took place in the microsomal fraction of the tumor [14]. Therefore, the decrease in the number of mitochondria in the Novikoff hepatoma does not alone explain the decreased phospholipid content. The decrease in the phospholipid content in the microsomes seems to be a more important factor in this respect.

The studies on the effect of fasting on the phospholipid content of the liver showed that even after 6 days fasting 50 per cent of the phospholipids were still present in the liver cell. On the other hand, the hepatoma cell contained only 30 per cent of the normal liver phospholipid content. Therefore, it is unlikely that anorexia and the resulting decreased food intake are responsible for the decreased phospholipid level of the Novikoff hepatoma.

It is interesting to note that the decrease in the phospholipid content characterized only neoplastic fast-growing liver, because no decrease is found in the fast-growing regenerating liver. In fact, the phospholipid content of the regenerating liver is increased at one day after hepatectomy.

The restoration rate of liver constituents agrees with previous data [10, 12]. The restoration rate of total liver phospholipids runs parallel with the restoration rate of liver weight, total liver nitrogen, total liver cellularity, except at 1 day after partial hepatectomy. The increased rate of restoration of total liver phospholipid at 1 day after hepatectomy is reflected in the high phospholipid content per unit tissue. The significance of this increase in the regenerating liver is not known.

SUMMARY

The phospholipid content of normal liver, Novikoff hepatoma and regenerating liver was investigated. The effect of 6 days fasting on the phospholipid content of normal rat liver was also studied.

1. The phospholipid content of Novikoff hepatoma was decreased to 44 and 73 per cent of that of the normal liver when expressed per wet weight or per mg nitrogen respectively. The phospholipid content in an average cell of the Novikoff hepatoma was only 32 per cent of that of an average normal liver cell.

2. The phospholipid content per unit was increased in the regenerating liver at 1 day after partial hepatectomy. At 3, 8, and 22 days after partial hepatectomy the phospholipid content per unit was the same in the regenerating liver as in the sham-operated controls.

3. The restoration of total liver phospholipid content was more rapid at 1 day after partial hepatectomy than the rate of restoration of liver weight, total liver nitrogen, total liver cell number, and liver/body weight ratio. However, at 3 days the total liver phospholipid restoration rate paralleled the restoration rate of the examined liver constituents and liver/body weight ratio.

4. The 6 days fasting resulted in a decrease of 50 per cent in the phospholipid content (per average cell) of rat liver.

5. The significance of the decrease of phospholipid content in the hepatoma and the changes in the regenerating liver were discussed.

The valuable technical assistance of Vilma Jansons is gratefully acknowledged.

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DEVELOPMENTAL CHANGES IN THE RESPIRATORY ACTIVITY OF RABBIT OVA

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The mechanisms whereby energy derived from metabolic reactions is channelled to drive specific processes in cleavage, differentiation and growth remain unknown. The elucidation of these mechanisms is of great fundamental importance. A review of the literature on energy exchange and enzyme development during embryogenesis has been recently compiled by Boell [2] with this point in mind. The developing fertilized ovum would appear to be the experimental object of choice for studies of this kind.

Since egg production in mammals is limited to small numbers and confined to a specific phase of the sexual cycle, extensive metabolic studies have been carried out only in sub-mammalian species. Limited investigations have been undertaken in the rat [4], the rabbit [7, 8 and 19] and the cow [6]. The effect of chemical agents upon oxygen consumption, cleavage and enzymes has been extensively studied in eggs of marine animals, mainly sea urchins (reviewed by Krahl [11]).

The purpose of this paper is to present the results of a study of the effect of metabolites, *in vitro*, on the respiratory activity of fertilized rabbit ova at various stages of development.

MATERIALS AND METHODS

Virgin rabbits weighing from 7 to 8 lbs. were purchased from Rockland Farms, N.Y. They were superovulated using sheep pituitary extract [16]. The animals were sacrificed about 20 to 115 hours *post coitum*. The tubes and uteri were flushed with Ca^{++}-free Krebs-Ringer Phosphate, pH 7.4 [21]; the abbreviation RP will be used to refer to this solution. In some cases 0.1 per cent glucose was added to the flushing medium. The abbreviation used for this solution will be RPG. Bovine hyaluronidase (Wyeth) was used to separate the ova from any attached cumulus cells. The ova, morulae and blastocysts were transferred to fresh RP or RPG before being placed in the Cartesian diver. In the case of late blastocysts of large diameter,

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they were punctured and the cellular mass freed from the surrounding zona pellucida or mucin coat. Rabbit serum was obtained from fresh blood collected by heart puncture.

The O_2 uptake of ova was measured with the Cartesian diver [3, 10, 13, 14]. This device is capable of measuring a gas exchange of less than one mμl. The ova plus the medium were pipetted into the divers with a braking pipette [10]. The Respiratory measurements were carried out at a temperature of 35°C. The volume of the medium ranged from 0.7 to 1.0 μl; the volume of the gas space ranged from 5 to 15 μl and each diver contained 7 to 15 early ova or morulae or one punctured blastocyst. Four series of experiments were carried out as follows.

I. O_2 uptake of freshly shed ova was determined in RPG with and without added serum. This was done in view of the fact that ova will cleave *in vitro* in the presence of rabbit serum, but not in RPG [9].

II. The effect of 10^{-2} *M* sodium pyruvate, 2×10^{-3} *M* phlorizin and 0.1 per cent glucose on O_2 uptake *in vitro* of freshly shed fertilized ova was studied. The effect of 10^{-3} *M* (approx.) sodium cyanide on early ova and large blastocysts was tested.

An attempt was made to find evidence for the existence of the tricarboxylic acid cycle in early ova, morulae and blastocysts. Malonate was added to the medium in an effort to inhibit succinic dehydrogenase and then fumarate was added to bypass the succinic dehydrogenase step.

The concentration of cyanide in the alkali was adjusted so that the final concentration in the medium at equilibrium would be about 10^{-3} *M*. The calculations used were those of Riggs [17] assuming the existence of ideal conditions.

III. O_2 uptake was measured after pre-incubating fertilized ova in RP for different lengths of time at varying temperatures. In this case the ova were transferred to a watch glass and placed inside a covered petri dish in the presence of wet paper to prevent evaporation; sterile precautions were taken. In these three series of experiments the gas phase was air.

IV. An attempt was made to measure glycolytic activity of ova, morulae and blastocysts using Ca^{++}-free Krebs–Ringer-Bicarborate, pH 7.4, as medium [21]. This medium will be referred to by the abbreviation RB. The gas phase was 95 per cent N_2 and 5 per cent CO_2. No attempt was made to remove the last traces of O_2 from the tank gas. Further, with the simple method of gassing used it is quite probable that a little air diffused into the divers before they were sealed off with paraffin oil and the flotation medium. The conditions therefore were not strictly anaerobic. In one case the O_2 content of a diver gassed under these conditions was measured by mixing a slightly acidified solution of pyrogallol with a solution of NaOH inside the diver. The mixing technique was that due to Anfinsen and Claff [1]. The oxygen content of the gas in the diver was found to be about 2 per cent.

RESULTS

The O_2 consumption of fertilized ova 34 hours *post coitum* ranged from 0.42 to 0.54 mμl/ovum/hr. In the presence of serum O_2 consumption ranged from 0.28 to 0.54 mμl/ovum/hr (Table I). There was no increase in respiratory

TABLE I

Effect of rabbit serum in vitro *on O_2 uptake of fertilized rabbit ova (34 hrs post coitum).* Gas phase: air.

Medium	O_2 uptake (mμl/ovum/hr) A	B	C
RPG	0.42	0.43	0.45
RPG + serum (1:1) [not corrected for apparent respiration of serum]	0.28	0.49	0.54
RPG + serum (1:1) [corrected for apparent respiration of serum]	0.21	0.49	0.44

rate in the presence of serum. When one considers the apparent O_2 uptake due to the serum, there is probably a slight decrease in two out of the three cases reported.

The question arose whether the zona pellucida and mucin coat presented a barrier to the diffusion of oxygen which might constitute a factor limiting the rate of oxygen consumption. An experiment was designed to test this possibility and the O_2 uptake was measured in two batches of punctured blastocysts. In one, the cellular mass was dissected free from the mucin coat. In the other, the mucin coat was left surrounding the collapsed cellular mass. There was no evidence of a decrease in O_2 consumption due to presence of the mucin coat.

As seen in Table II, respiration was markedly depressed in the presence of cyanide at the two stages tested, early ovum and large blastocyst. A high concentration of cyanide (1.0 *M*) must be present in the alkali neck seal (which is 0.1 *N* NaOH). Without cyanide in the alkali, the cyanide in the medium distills off into the alkali and is thus removed from the medium [12]. We have found that this results in resumption of egg respiration which illustrates the well-known reversibility of the cyanide effect. The presence of 2×10^{-3} *M* phlorizin did not inhibit O_2 uptake of freshly shed fertilized ova. The addition of 10^{-2} *M* sodium pyruvate caused little or no increase in the respiratory rate. The addition of glucose had no apparent effect on O_2 uptake. The addition of malonate *in vitro* had no effect on the O_2 uptake of the morulae and early blastocyst. The further addition of fumarate caused no significant change in respiratory rate. We could therefore not obtain evidence for the presence of the tricarboxylic acid cycle. Malonate may fail

TABLE II

O_2 uptake of fertilized ova in different media.

Gas phase: air.

Developmental stage		Medium in the divers		Average O_2 uptake
Morphology	Hrs post coitum	Basic medium	Added substances (*M*)	mμl/ovum/hr
2–8 cells	24–30	RPG	None	0.41
			10^{-3} *M* NaCN (appr.)	0.02
			10^{-3} *M* phlorizin	0.41
		RPG	None	0.56
			2×10^{-3} *M* Na fluoride	0.47
Morulae	68	RP	None	0.48
			10^{-2} *M* malonate	0.42
			10^{-2} *M* malonate plus 10^{-3} *M* fumarate	0.47
Blastocysts	78	RP	None	1.71
			10^{-2} *M* malonate	1.69
			10^{-2} *M* malonate plus 10^{-3} *M* fumarate	1.74
„	88	RP	None	2.92
			10^{-2} *M* malonate	2.36
			10^{-2} *M* malonate plus 10^{-3} *M* fumarate	2.70
„	115	RPG	None	78.00
			10^{-3} *M* NaCN (appr.)	0.00

to penetrate the cell membrane(s) or, possibly, other oxidative pathways exist which can come into play when succinic dehydrogenase is blocked.

Pre-incubation of freshly shed ova or blastocysts in RP for some 90 to 180 minutes did not seem to depress significantly the endogenous O_2 consumption (Table III). Further, the presence of either glucose or pyruvate did not affect the O_2 uptake of the pre-incubated eggs.

Preliminary experiments were carried out to measure the gas exchange of fertilized ova under low O_2 tension in a bicarbonate buffer as described above (Experimental Series IV). In the absence of exogenous glucose there was a gas uptake observed in all stages tested. With 0.1 per cent glucose present in the medium the gas uptake was unchanged in the earliest stages

TABLE III

Effect of pre-incubation O_2 uptake of fertilized ova.

Incubating medium: Ca^{++}-free Krebs–Ringer phosphate, pH 7.4. Gas phase: air.

Developmental stage		Pre-incubation of ova		Metabolites added to RP in diver	Average O_2 uptake mμl/ovum/hr
Morphology	Hrs post coitum	$T°C$	Time (min)		
		23	120	None	0.45
		23	120	0.1 % glucose	0.49
		23	120	10^{-2} *M* pyruvate	0.47
2–4 Cell	20–28	29	90	None	0.45
		29	90	0.1 % glucose	0.42
		29	90	10^{-2} *M* pyruvate	0.41
		29	180	None	0.39
		29	180	0.1 % glucose	0.59
		29	180	10^{-2} *M* pyruvate	0.53
		37	150	None	1.84
Blastocyst	108	37	150	10^{-2} *M* pyruvate	2.42

(2–8 cells), but late morulae and blastocysts showed a gas evolution indicative of lactic acid production. These results indicate that (*a*) there is little or no endogenous glycolysis in any of the stages tested; (*b*) the addition of glucose to the medium reveals that at the 2 to 8 cell stage there is little or no capacity to carry out glycolysis and that this capacity appears to have been developed in the late morula and is further increased in the blastocyst. More recent experiments under strictly anaerobic conditions (to be published separately) have confirmed these preliminary findings.

DISCUSSION

The inhibiting effect of cyanide may be presumed to act through the cytochrome oxidase system although that system has not been directly demonstrated in the mammalian ovum. Phlorizin and fluoride were tested for their possible effect on respiration through their action on the glycolytic system. There is no significant effect by either. The indication is that glucose is not an obligatory substrate for respiratory activity in this tissue. Externally supplied pyruvate or glucose caused little or no increase in respiration either with or without pre-incubation. These results suggest the existence of an intracellular store of nutrients. That this store is not glucose is further

indicated by the observations made during measurements of glycolytic activity. Where this activity was clearly observed it was dependent on the presence of exogenous glucose. It was thought that the ova could be depleted of this nutrient reserve by pre-incubating the ova in RP. Subsequent measurement of O_2 uptake with and without added metabolites revealed no significant effect on O_2 consumption that could be due to substrate depletion. Superficial microscopical examination of the ova after the pre-incubation period revealed that the morphological appearance was essentially normal. Hafez [9] has tested the viability of the ova after incubating in a glucose-free Ringer Phosphate kept under sterile conditions at 37°C for some 90 to 180 minutes. The transplantation of the ova (30, 60 hrs post coitum) into recipients at a corresponding stage of pseudopregnancy did not result in implantation. Ova will not cleave in RPG, therefore the ovum after or during incubation in Ringer is not a developing organism but a resting one and interpretation of results must be made with this in mind. Since the ova will not develop in RPG but do so in serum [9] measurements of O_2 uptake were carried out in a medium containing serum. There was no marked change in O_2 uptake caused by the presence of serum. There was a decrease in the respiratory rate in one or possibly two cases out of three.

The energy requirement for growth and differentiation has been estimated by Butler [5] to be a small fraction of the total energy production of the cells. Thus the developing ovum can synthesize living structures with a fairly modest expenditure of metabolic energy. Warburg's observation (amply confirmed) that fertilization results in a marked increase in the O_2 uptake of sea urchin eggs [15, 18, 20, 22, 23] led to the notion that growth and differentiation account for most of the energy metabolism manifested as O_2 uptake. Later work revealed that the sea urchin egg is rather a special case. In other species there are examples of increases, decreases or no changes in oxygen uptake after fertilization [23]. Fridhandler *et al.* [7] found no increase in O_2 uptake after fertilization of rabbit ova. The interpretation of these results is made difficult by the fact that unfertilized rabbit ova had a small variable quantity of cells of follicular origin still attached even after treatment with hyaluronidase.

It is interesting to note that definite glycolytic activity under the present experimental conditions has been observed only at the morula stage and in the blastocyst. This may illustrate the development of an enzyme system of the fertilized ovum in the pre-implantation stages. On the other hand, the possibility must be entertained that changes in membrane characteristics of the developing egg are the factors affecting these observations.

SUMMARY

Oxygen consumption of fertilized rabbit ova in Ca^{++}-free Ringer-Phosphate, pH 7.4 (RP) at various stages of development has been measured in the Cartesian diver in the presence of certain metabolites and inhibitors. Cyanide exerted a marked inhibitory effect on respiration presumably through the cytochrome oxidase system. Fluoride, phlorizin, malonate, malonate-fumarate combinations, pyruvate and glucose had no marked effect on respiratory rate.

The presence of homologous serum did not increase the rate of respiration. Indeed, in one out of three cases there appeared to be a definite inhibition of O_2 uptake.

The mucin coat of large blastocysts does not seem to be a limiting barrier to O_2 consumption by blastocyst tissue.

Pre-incubation of ova, morulae and blastocysts in RP for $1\frac{1}{2}$ to 3 hours resulted in no apparent decrease in endogenous respiratory rate. After such a pre-incubation, the presence of metabolites such as glucose or pyruvate had no significant effect on O_2 consumption. The results suggest the availability of an intracellular store of substrate in the fertilized developing egg which cannot be exhausted by the pre-incubations tested. That this store is not glucose or a glucose precursor in late morulae and in blastocysts is indicated by the following: preliminary experiments have shown that late morulae and blastocysts, but not freshly shed ova, display glycolytic activity only in the presence of exogenous glucose. No evidence for glycolytic activity in early ova (1 to 16 cells) has been found. These data probably reflect the emergence of an enzyme complex during development in the fertilized rabbit ovum.

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EFFECT OF 20-METHYLCHOLANTHRENE ON DNA SYNTHESIS IN MOUSE PROSTATES GROWN *IN VITRO*

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Mouse prostate glands when grown by the watchglass technique preserve their histological structure and function *in vitro*. They consist of alveoli lined with a row of secretory epithelium surrounded by strands of connective tissue. In previous work [2, 3, 4] it was found that the addition of 20-methylcholanthrene (MC) to the medium stimulates mitosis of the alveolar epithelium after a few days' treatment, and causes hyperplasia and squamous metaplasia characterized by cellular enlargement and polyploidy. At the same time the growth of the connective tissue is inhibited.

The present study was undertaken to find whether these morphological changes were correlated with changes in the synthesis of desoxyribonucleic acid (DNA); to investigate this problem, we decided to label the nucleic acids in carcinogen treated and control cultures with [8-^{14}C]-adenine, and to prepare autoradiographs of sections containing mixed nucleic acids and of others containing DNA only.

MATERIALS AND METHODS

The ventral prostate glands of C_3H mice were grown on clots consisting of chick plasma, horse serum and chick embryo extract. MC suspended in serum was added to the medium, in a concentration of 4 μg/ml. The cultures were transferred to a new clot and fresh carcinogen every three days.

The explants were labelled after 4, 7 and 10 days' treatment with MC by the addition of one drop of a Tyrode solution containing [8-^{14}C]-adenine in a concentration of 50 μc/ml. After 30 seconds the remaining fluid was pipetted off and the cultures were returned to the incubator for periods of 5 or 24 hours. They were then fixed in acetic acid alcohol followed by formol saline and embedded in paraffin wax. Sections were cut at 5 μ. After the paraffin had been removed one half of the slides were treated with ribonuclease or with cold perchloric acid, and the other half were kept in veronal buffer without ribonuclease, or in distilled water. Autoradiographs were prepared by the stripping film technique. All preparations were stained with carmalum or haematoxylin. Altogether 72 explants were studied.

RESULTS

Autoradiographs of sections prepared by the methods described above, can be interpreted as being due in unextracted slides to [8-^{14}C]-adenine incorporated into RNA and DNA synthesized between the time of application of the tracer and fixation, and in slides extracted with ribonuclease or cold perchloric acid, to labelled DNA [6].

Distribution of adenine in controls

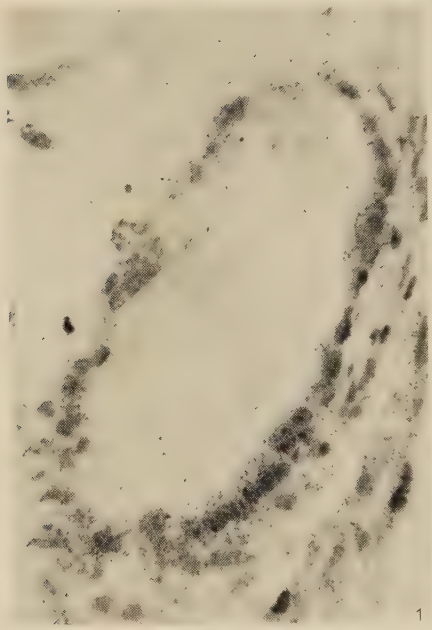
DNA not extracted.—In all explants grown in normal medium autoradiographs (ARG) of sections fixed 5 hours after addition of the tracer show relatively heavy uptake in the nuclei of most epithelial cells and fibroblasts, particularly near the periphery of the explant. The cytoplasmic ARG is invariably weaker than that of the nuclei; traces of activity are occasionally found in the cytoplasm of unlabelled nuclei. Cells extruded into the secretion are unlabelled.

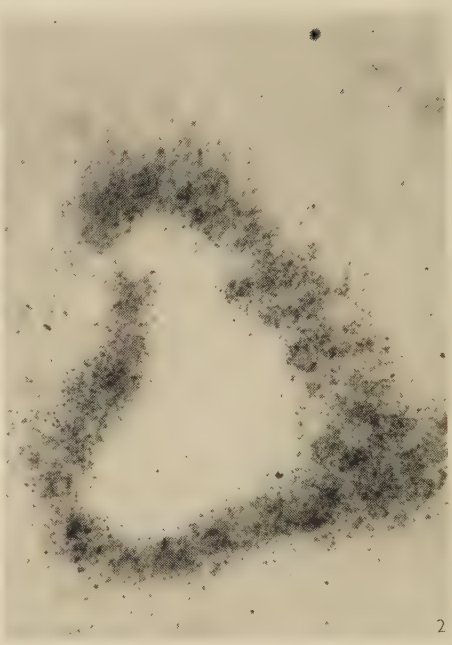
Twenty-four hours after application of the tracer, the ARG is denser over all cells except those extruded into the secretion, which are still negative, though there is some labelling of the secretion itself. The cytoplasm of all metabolizing cells is much more heavily labelled than at 5 hours and in many cells the ARG over the cytoplasm is heavier than that over the nucleus.

RNA extracted. –A number of positive nuclei, including some in division, are found in preparations fixed 5 hours after the application of adenine (Fig. 1). Usually positive nuclei are more abundant in the epithelial cells and fibroblasts near the edge of the explant than towards the centre. Nuclei scored as positive show autoradiographs of from 10 to 50 grains per nucleus after 20 days' exposure. Some cells seen in division are weakly labelled, and others are unlabelled. The general distribution of labelled nuclei is the same in preparations fixed after 24 hours in labelled medium. The nuclei with the heaviest ARG now show up to 70 grains, but the variability in the strength of ARG is less than at 5 hours, i.e. at the earlier time few heavily labelled and many weakly labelled nuclei are found, whilst later many positive nuclei have an intense ARG. At 24 hours all nuclei seen in division are labelled.

Distribution of adenine in cultures treated with 20-methylcholanthrene

RNA not extracted.—Five and 24 hours after the application of labelled adenine, the distribution in the epithelial cells lining the alveoli is similar to that in the controls (Fig. 2). A few well labelled fibroblasts are found after





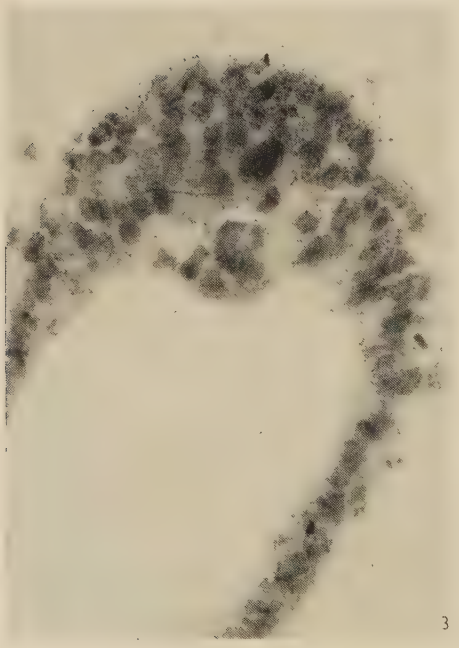


Fig. 1.—Stripping film autoradiograph of a section of a mouse prostate explant grown for 11 days in normal medium. [8-^{14}C]-adenine added 24 hours before fixation. Extracted for 18 hours with 10 per cent perchloric acid at 4°C. Haematoxylin and eosin. Note the labelling of nuclei in epithelium and connective tissue.

Fig. 2.—Stripping film autoradiograph of a section of a mouse explant prostate grown for 9 days in medium containing 4 μg/ml of 20-methylcholanthrene, followed by 1 day in normal medium. [8-^{14}C]-adenine added 5 hours before fixation. No extraction. Haematoxylin and eosin. Many epithelial cells are labelled but not those of the connective tissue.

Fig. 3.—Same explant as Fig. 2 but extracted for 18 hours with 10 per cent perchloric acid at 4°C. Only the labelled DNA is shown.

4 days' treatment with the carcinogen; at 7 and 10 days all fibroblasts are unlabelled or weakly labelled.

RNA extracted with cold perchloric acid.—After 5 hours the nuclei are labelled in a number of epithelial cells (Fig. 3); some cells seen in division are labelled, others are negative. Labelled nuclei show up to 50 grains per nucleus after 5 hours' growth in labelled medium and up to 70 after 24 hours. A few very heavily labelled nuclei, presumably of polyploid cells, are seen after 9 days' treatment with the carcinogen. Some nuclei of fibroblasts are positive after 4 days' treatment, but at later stages all of them are negative, a fact which is probably correlated with the previously reported inhibition of growth in the connective tissue of methylcholanthrene treated explants.

Numerical results

Nuclei were scored as positive when the number of grains in the autoradiograph were well above background, in most slides 8 or more. Only the alveoli showing fair activity were counted. The index of DNA synthesis (SI) is expressed as

$$\mathrm{SI} = \frac{\text{positive nuclei}}{\text{all nuclei}} \times 100.$$

Mitotic figures from metaphase to telophase in alveoli synthesizing DNA were counted on autoradiograph preparations stained with haematoxylin and eosin. They are expressed as

$$\mathrm{MI} = \frac{\text{mitotic figures}}{\text{all nuclei}} \times 100.$$

The numerical results are summarised in Table I and Fig. 4 and will be referred to in the discussion.

DISCUSSION

It can be concluded that in many cells the nuclear ARG at 5 hours in unextracted slides is due to labelled RNA, which is removed by cold perchloric acid. The finding that at 5 hours the nuclear RNA in the control explants is more heavily labelled than the cytoplasmic, whilst at 24 hours the cytoplasmic ARG is often more intense than the nuclear, agrees with the results of Smellie *et al.* [7, 8] on the incorporation of ^{32}P into the nucleic acids of various tissues *in vivo*, and suggests some movement of labelled material

Table I

Percentage of labelled and mitotic cells in prostatic epithelium after extraction with cold PCA.

5 hrs adenine ...	SI (%)	MI (%)	24 hrs adenine ...	SI (%)
M2, 4 days	43	3.47	M1, 5 days	58
Co2, 4 „	45	1.20	Co1, 5 „	49
M4, 7 „	59	4.79	M3, 8 „	70
Co4, 7 „	41	2.51	Co3, 8 „	69
M5, 10 „	60	5.74	M6, 11 „	70
Co5, 10 „	44	0.33	Co6, 11 „	50
Mean Co	43	1.34	Mean Co	56

Co: Controls grown in normal medium.
M: Experimental cultures grown in medium containing methylcholanthrene.
SI (synthetic index): percentage of epithelial cells which synthesized DNA. Standard error ± 4 per cent.
MI: Mitotic index (meta- telophase) counted over H & E stained ARG's in epithelia showing DNA synthesis. Standard error ± 0.5 per cent.

from the nuclei in our experiments. It is therefore likely that at least some of the labelled RNA in the cytoplasm is derived from nuclear RNA.

The observations on control cultures extracted with cold perchloric acid show that DNA is synthesized by epithelial cells as well as by fibroblasts.

Since at 5 hours only some of the mitotic cells are labelled and these weakly, while some interphase nuclei give an intense ARG, it can be assumed that about 2–3 hours elapse between the end of DNA synthesis and the onset of metaphase. Thus cells which begin to synthesize DNA soon after adenine is added, have 5 hours in which to incorporate it into DNA; at the moment of fixation, therefore, they will be heavily labelled but will not have entered mitosis; on the other hand, cells which are nearing the end of the period of DNA synthesis when placed in contact with the labelled adenine, have only a short time in which to incorporate the nucleic acid, but they may have begun division by the time the culture is fixed.

Since the number of grains for heavily labelled nuclei of epithelial cells only increases from 50 to 70 between 5 and 24 hours, DNA synthesis probably lasts for not more than about 7 hours, since a nucleus showing 50 grains at 5 hours has synthesized for 5 hours at the most.

It can be concluded that the duration of the stages of the mitotic cycle which can be estimated from our results, is similar to that in the controls.

Table I and Fig. 4 show that the mitotic index in the prostatic epithelium

is doubled after 4 days' treatment with methylcholanthrene whereas the synthetic index is almost unchanged. After 7 and 10 days' treatment the mitotic index is 3 to 4 times that of the average value for controls, yet the index of DNA synthesis has only increased by approximately 40 per cent.

To obtain the index of DNA synthesis, positive nuclei, irrespective of the strength of the ARG, were scored against the unlabelled nuclei; it is therefore

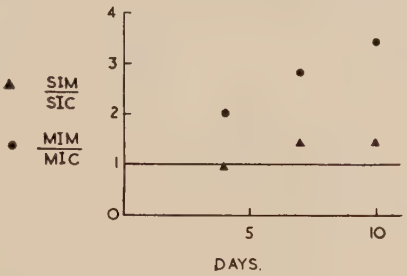


Fig. 4.—The synthetic index after treatment with 20-methylcholanthrene divided by that in controls (▲), and the mitotic index after treatment divided by that in controls (●). The carcinogen has caused only a slight rise in the synthetic index, but a great increase in the mitotic index.

an index of the number of cells synthesizing DNA and not of the total amount of DNA synthesized. It could be assumed that synthesis is slower in controls and therefore a relatively larger number of cells show evidence of DNA synthesis, but this is disproved by the fact that the number of grains per positive nucleus, which is proportional to the amount of ^{14}C-adenine incorporated, is no higher in the treated than in the control cultures. The increased mitotic index cannot be due to a prolongation of mitosis, because cell proliferation is undoubtedly higher in the epithelial tissues of treated than of untreated cultures [2, 3, 4].

Since the number of cells synthesizing DNA in treated cultures is sufficient to maintain a high mitotic index, it has to be assumed that not all DNA synthesis in controls leads to mitosis. This view is supported by previous [5] observations of nuclear autoradiographs in extracted sections of the tracheal epithelium and the seminal vesicle of mice injected with [8-^{14}C]-adenine. The mitotic index in these tissues is too low (of the order of 1:100,000 [1]) to account for the number of cells which are actively synthesizing DNA at a rate comparable with that in dividing cells. It may therefore be assumed that nuclei in some non-dividing tissues synthesize DNA for metabolic purposes. It is suggested that cells which would normally differentiate into secretory elements and which synthesize DNA in the explanted prostate are forced into mitosis by the action of 20 methylcholanthrene. This may possibly be brought about by binding of a specific protein or enzyme system necessary for the regulation of cell differentiation and cell multiplication.

SUMMARY

Ventral prostate glands of C_3H mice were grown *in vitro* in normal medium and in medium containing 4 μg per ml of 20-methylcholanthrene. The explants were labelled with [8-^{14}C]-adenine after 4, 7 and 10 days' treatment and fixed 5 or 24 hours later.

Autoradiographs of unextracted slides or slides from which the RNA had been removed with ribonuclease or cold perchloric acid were prepared.

Untreated sections of controls show incorporation of adenine in both epithelium and fibroblasts. In explants exposed to the carcinogen the distribution of labelled cells in the alveolar epithelium is similar to that in the controls; labelled fibroblasts are still found after 4 days' treatment, but at 7 and 10 days they are unlabelled.

Autoradiographs of sections treated with ribonuclease or cold perchloric acid can be assumed to be due to labelled DNA. In controls, epithelium and fibroblasts are labelled; in MC treated explants epithelial cells only are labelled while DNA synthesis by fibroblasts is inhibited after more than 4 days' treatment.

Cell counts show that after 4 days' treatment with the carcinogen the percentage of epithelial cells synthesizing DNA is equal to that in controls although the mitotic index is doubled; after 7–10 days the percentage of synthesizing cells increases by 40 per cent in the treated explants, but the mitotic index rises to 3–4 times the control value. It is suggested that some cells in the controls synthesize DNA without proceeding to division and that these cells are forced into division by 20-methylcholanthrene.

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INFLUENCE OF INTRAPERITONEALLY INJECTED HOMOGENATES OF THE OUTER ORBITAL GLAND AND THE LIVER ON RNA AND DNA IN THESE ORGANS IN RATS[1]

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Injections of tissue homogenates into laboratory animals have revealed organ-specific mitosis stimulations [1, 2, 26, 32, 36]. The activity decreases according to the age of the donor [28] and increases at the regeneration stage of the organ [2]. After injury to organs occurring in pairs, the untreated organ has shown signs of increased mitotic activity [10, 27]. The background of both phenomena is obviously the same, viz., activation and release of growth-stimulating agents.

The humoral action of the growth-stimulating agents is revealed, not only by experiments performed on organs occurring in pairs, but also by mitotic stimulation observed in one parabiotic rat when the other has been hepatectomized [3, 37]. A similar effect has also been noted by injecting serum after hepatectomy [11]. Tissue culture studies have shown better growth in serum from hepatectomized rats compared with growth of liver cells in normal serum [12].

The nature of the mitosis-stimulating agents is, as far as we know, not yet explained. The outer orbital gland of the rat is the organ most studied in our laboratory. The agent of this organ is thermostable, effective after drying and operates in 6–8-week-old rats after an intraperitoneal injection of 0.2–1 mg of tissue from 2-week-old rats [29].

An organ-specific increased uptake of P^{32}, which is simultaneously correlated to the mitotic frequency, has been found in the outer orbital gland of rats which were injected with homologous homogenates 48 hours and P^{32} one hour before decapitation [15].

It may be possible that the action in some way takes place through the nucleic acids or that it influences the nucleic acid synthesis. Therefore we decided to study whether the homogenates made from the following organs influence the RNA and DNA of these organs: the orbital gland of the 2-week-

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old rat at an active stage of growth; the liver of rats at different ages; and the liver in a state of regeneration after partial hepatectomy. The last-mentioned organ was included because nucleic acids have been studied rather extensively in hepatectomized rats. By using regenerating tissue, which is known to stimulate the mitotic acitivity when injected as homogenates [2], we made attempts to study the possible changes caused by it in RNA, DNA and in the mitotic frequency.

METHODS

Wistar rats, kept under similar conditions and fed with a standard diet, were used in the experiments.

The rats were injected intraperitoneally with orbital gland homogenates, normal liver homogenates, and homogenates prepared from the regenerating liver. The concentration of the orbital gland homogenate was 1 mg/ml of distilled water, the amount injected 0.4 ml, and the homogenate was prepared from a 2-week-old rat. The normal liver homogenate was prepared from the liver of 1-day-old and 6-week-old rats, its concentration being 6 mg/ml and the amount injected 0.5 ml. The concentration of the homogenate prepared from the regenerating liver was similar to that prepared from the normal liver. It was prepared two days after hepatectomy. The control animals were injected 0.4 and 0.5 ml of distilled water or saline solution, respectively. The homogenates and solutions were not prepared sterilely. The homogenates were made at room temperature by crushing the organs in a glass homogenizer.

Before the injection, one of the outer orbital glands of the animals was removed and a biopsy specimen was taken from the liver for nucleic acid determinations. At the same time a histological specimen was fixed in Bouin's solution for counting the mitoses. Injections were performed 3 days after the operation. The rats were decapitated 1, 2 and 7 days after the injection and the nucleic acid determinations were made from the corresponding organs. Using this method the possible changes in the nucleic acid concentrations could be observed individually.

The outer orbital gland is situated just under the skin in front of the ear and is easy to extirpate. The specimens from the liver were taken through incision, about 2 cm long, made in the abdominal wall. Before the experiment the specimen was removed from the left lobe and thereafter from the median lobe. The mean weight of the specimens taken before the experiment was 150 mg. The operation of the outer orbital gland and the liver before the injections was performed under ether anesthesia.

The partial hepatectomy was performed under ether anesthesia. Through a ca. 3 cm long incision, the left and median lobes of the liver were removed. The weight of the removed part was about 30 per cent of the total weight of the liver.

The specimens were treated at about +5°C immediately after the operation by using Schneider's method [21]. The tissue residues were dried at +4°C in a vacuum dessicator and were kept thereafter in a refrigerator. The extraction of the nucleic acids from the tissue residue was performed with the aid of Ogur–Rosen's method [20].

The nucleic acid determinations were made spectrophotometrically by using orsinol reagent [5] for the RNA and diphenylamine for the DNA [9, 22]. The concentration of RNA and DNA is given as per cent of the dry weight.

RESULTS

Controls

Basic control

Before the main experiment, we studied whether the removal of the one of the outer orbital glands influences the nucleic acid concentration of the other and whether taking of the specimen from the liver influences its nucleic acid concentration.

Outer orbital gland.—The right orbital gland was removed under ether anesthesia from 4 adult animals, which were decapitated 2 and 7 days later when the differences of the percentage values of RNA ranged from -0.23 to $+0.14$, those of DNA ranging between -0.10–$+0.23$. No difference was noted between the different observation periods.

Liver.—Biopsy specimens of the liver were taken under ether anesthesia from 3 rats, which weighed 100, 75 and 80 g. The specimens weighed 188, 257 and 286 mg. These weights were greater than those used later in the main experiments. The animals were decapitated 2 and 7 days after the operation when the differences of the percentage values of RNA ranged from -0.03 to $+0.32$, those of DNA ranging from -0.19 to ± 0.

After the removal of the orbital gland and the liver specimen, no significant differences were observed in the nucleic acid concentration. One animal only, 2 days after the removal of the specimen, showed a difference of $+0.32$ for RNA of the liver.

Cross controls

The group included the control animals used in the main experiments, 12 in total. Three days after the removal of the right orbital gland and the liver specimen, the animals were injected with saline solution or distilled water and were decapitated 1, 2 and 7 days after the injections. The procedure caused no changes in the nucleic acid concentration of the orbital gland and the liver (Table I).

In the entire control series, the standard deviations of per cent changes were: the outer orbital gland, RNA 0.18, DNA 0.17; and the liver, RNA 0.16, DNA 0.17.

TABLE I

The means and standard deviations of the differences of percentage values in the nucleic acid concentration of the orbital gland and the liver of the control animals.

Time of influence of injections (days)	Outer orbital gland						Liver					
	RNA			DNA			RNA			DNA		
	No. of rats	Mean diff.	SD	No. of rats	Mean diff.	SD	No. of rats	Mean diff.	SD	No. of rats	Mean diff.	SD
1	4	+0.13	0.16	4	−0.07	0.19	3	−0.14	0.13	3	−0.05	0.26
2	4	−0.06	0.09	4	−0.03	0.11	3	+0.19	0.11	3	−0.02	0.02
7	4	−0.04	0.21	4	−0.05	0.17	3	+0.11	0.06	3	−0.05	0.18

On the basis of these values, the significance of the results from the main experiments was calculated by using the *t*-test.

Influence of Tissue Homogenates of Outer Orbital Gland and Liver from Young Rats

Homogenates from the outer orbital gland

EXP. 1.—The group consisted of 11 animals, 3 of them controls. Their weights ranged between 98–144 g. Three days after the operation, 8 rats were injected with 0.4 ml of orbital gland homogenate and the controls 0.4 ml of distilled water. The animals were decapitated 1, 2 and 7 days after the injections.

The orbital gland: A significant increase occurred in the RNA and DNA concentrations (Table II). The concentration of the RNA was high 1 and 2 days after the injection, while after 7 days no difference could be observed as compared with the controls. The concentration of DNA was, however, high even 7 days after the injection.

The liver: No effects could be observed. The mean differences were similar to those of the controls (Table II).

EXP. 2.—This was a renewal of the previous test, but as the orbital homogenate did not influence the nucleic acid concentration of the liver, the specimens were taken from the orbital gland only. The group consisted of 8 animals, 3 of them controls. Their weights ranged between 66–127 g.

As compared with the results of the previous experiment, the only difference was that the concentration of RNA was even after 7 days somewhat higher than the original value, viz. +0.50 per cent, $P < 0.05$, while the concentration of DNA had decreased to the original level (Table II, Diagram 1).

TABLE II

Mean differences and significance of results.

Time of influence of injections (days)	Outer orbital gland						Liver					
	RNA			DNA			RNA			DNA		
	No. of rats	Mean diff.	*P*	No. of rats	Mean diff.	*P*	No. of rats	Mean diff.	*P*	No. of rats	Mean diff.	*P*
Exp. 1												
1	1	+1.07	<0.001	2	+0.48	<0.01	2	+0.06	>0.05	2	+0.09	>0.05
2	3	+1.12	<0.001	3	+0.71	<0.001	3	−0.08	>0.05	3	−0.02	>0.05
7	3	+0.06	>0.05	3	+0.62	<0.001	3	−0.10	>0.05	3	−0.08	>0.05
Exp. 2												
1	2	+1.11	<0.001	2	+0.68	<0.001						
2	2	+1.05	<0.001	2	+1.10	<0.001						
7	1	+0.50	<0.05	1	+0.10	>0.05						
Exp. 3a												
1	2	−0.05	>0.05	2	+0.39	<0.01	2	+0.42	<0.01	2	+0.03	>0.05
2	3	−0.08	>0.05	3	+0.42	<0.01	3	+0.02	>0.05	3	−0.04	>0.05
7	3	+0.16	>0.05	3	+0.46	<0.001	2	+0.12	>0.05	2	−0.04	>0.05
Exp. 3b												
1							2	−0.10	>0.05	2	+0.04	>0.05
2							3	−0.08	>0.05	3	+0.10	>0.05
Exp. 4												
1	3	−0.18	>0.05	3	−0.03	>0.05	3	+0.95	<0.001	3	+0.003	>0.05
2	3	+0.08	>0.05	3	−0.03	>0.05	3	+1.55	<0.001	3	+0.03	>0.05
7	3	−0.14	>0.05	3	+0.09	>0.05	3	+1.17	<0.001	3	±0.00	>0.05
Exp. 5												
1							3	+0.07	>0.05	3	+0.08	>0.05
2							4	+0.37	<0.001	4	+02.5	<0.05
Exp. 6												
1							4	+0.40	<0.001			
2							5	+0.53	<0.001			

Liver from the new-born rats

EXP. 3*a*.—The group consisted of 11 animals, 3 of them controls. They weighed 54–94 g. The operation and decapitation were performed similarly to the previous experiments. Eight animals were injected 0.5 ml of the liver homogenate of 1-day-old rat and the controls 0.5 ml of saline solution.

The orbital gland: No changes occurred in the concentration of the RNA. After 7 days, one value was + 0.70 per cent, but the mean of 3 animals was + 0.16, $P > 0.05$. The DNA concentration, on the other hand, had increased throughout, even after 7 days the mean difference was + 0.46, $P < 0.001$ (Table II).

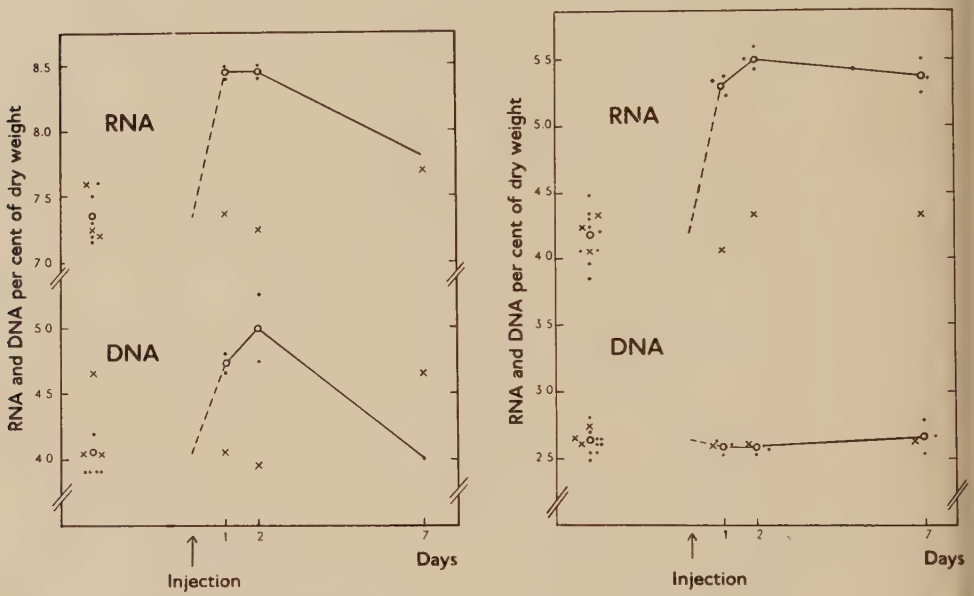

Fig. 1 (*left*).—Experiment 2. Influence of the outer orbital gland homogenate on the RNA and DNA concentration of the outer orbital gland. × controls; ○ mean of two or more percentage values. The high DNA values in a control at start and after 7 days are from the same animal. Fig. 2 (*right*).—Experiment 4. Influence of the homogenate from regenerating liver on the RNA and DNA concentrations of the liver. × controls; ○ mean of three or more percentage values.

The liver: The concentration of the RNA was increased in one case only. After 1 day, a value of + 0.88 per cent was obtained. The mean difference of percentage values was thus + 0.42, $P < 0.01$, in this group of 2 animals (Table II). No significant differences occurred in the DNA values.

Liver from 6-week-old rats

Exp. 3*b*.—In connection with hepatectomy (Exp. 5), the removed specimen was placed in a deep-freezer (− 20°C) for 2 days. A homogenate was prepared from this specimen and was injected into 5 rats, which weighed 95–113 g. The homogenate did not change the nucleic acid concentration of the liver 1 and 2 days after the injections.

Influence of Homogenates Made from Regenerating Liver Taken Two Days after Hepatectomy

Exp. 4.—The group consisted of 12 animals, 3 of them controls. They weighed 45–63 g. Nine animals were injected 0.5 ml of homogenate prepared from the regenerating liver and the controls 0.5 ml of saline solution.

The orbital gland: No influence was observed.

The liver: The increase in the concentration of RNA was highly significant, being great even 7 days after the injection. No changes occurred in the concentration of DNA (Table II, Diagram 2).

Exp. 5.—In this experiment the influence on the nucleic acids of the liver was studied. The group consisted of 12 animals, 5 of them controls. They weighed 83–127 g. Decapitation was performed 1 and 2 days after the injections. Seven animals were injected with 0.5 ml of homogenate prepared from the regenerating liver and the controls with 0.5 ml of homogenate made from the normal liver of 6-week-old rats (cf. Exp. 3*b*).

The RNA concentration of the liver showed no increase after 1 day (the mean of the differences was $+0.07$, $P > 0.05$), while differences occurred after 2 days as compared with the controls. In a group of 4 animals, the mean was $+0.37$, $P < 0.001$. Contrary to the previous experiment, a slight increase was also observed in the DNA concentration after 2 days, the mean of the differences being $+0.25$, $P < 0.05$.

Exp. 6.—The influence on the RNA of the liver was studied. The group consisted of 9 animals which weighed 63–114 g. No control animals were included, as the original percentage values of RNA were considered controls.

A significant increase occurred in the RNA concentration both 1 and 2 days after the injections. The mean differences were $+0.40$ and $+0.53$ ($P < 0.001$).

CONCLUSIONS

The original purpose of the present work has been to study the influence of injected homogenates both on the nucleic acid concentration and on the mitoses. We did not realize our plan completely, for it became evident that in cases without mitosis stimulation there might, nevertheless, occur a significant increase in RNA and DNA. The increase in the DNA concentration is probably due to the fact that the injected homogenate also influences the nuclear class series, as has been shown in our laboratory [33]. The growth-stimulating influence of tissue homogenates should therefore be studied from

a wider cytological point of view, taking into consideration the endomitotic activities, the density of cells, and the number of binuclear cells. Experiments in this field are in progress.

When evaluating the results it might seem, perhaps, that there could be direct resorption of the injected homogenate into the organ in question. However, as the influence has been induced by injecting very small amounts and the increase is in no correlation to smallness of the amounts, there must be direct influence on the nucleic acid synthesis. Nevertheless, we are not able to explain this phenomenon on the basis of the present study.

Our results refer to a certain specificity. This concerns, above all, the homogenate of the orbital gland, the influence of which is specifically directed to RNA and DNA of the orbital gland. Moreover, a tissue-specific influence was always obtained to the RNA with both homogenates in the cross experiments.

According to Chargaff [6], DNA is species specific and RNA tissue specific. He came to this conclusion on the basis of purine and pyrimidine base analyses which he performed with nucleic acids of different origin. Species specificity of DNA is also referred to in a paper which shows that by using the eggs of *Amphibia* and *Asterias*, DNA isolated from the liver of the same species inhibits the embryonic development at a certain stage, while DNA of the liver of cattle has no influence [19]. It has also been shown that DNA isolated from the liver decreases the mitotic rate of the regenerating liver, while DNA of the thymus has no influence [7].

The tissue specificity of the RNA does not seem to be absolute. Strict organ specificity of the gastric mucosa has been shown with the aid of serological reactions [35], but the same method has also shown that the RNA of the kidney and liver has partly a common and partly a tissue-specific antigenic structure [18].

It has been indicated in the present work that the field of action of the liver homogenate is larger than that of the homogenate of the outer orbital gland. The homogenate prepared from the regenerating liver influenced the RNA concentration of the liver only, while the liver of the new-born rat increased the DNA concentration of the orbital gland without having any influence on the liver.

Previous work performed in our laboratory equally refers to the relative specificity of the liver. We have found, for instance, that the ribonuclease of the orbital gland is specific and influences the RNA of the orbital gland only, the field of action of the ribonuclease of the liver being larger [30]. In the studies on the uptake of P^{32} it has become evident that the liver

homogenate causes a slight mitosis stimulation in the orbital gland, while the orbital homogenate does not influence liver [15].

The increase in the concentration of the RNA does not necessarily argue in favor of growth stimulation, because the RNA concentration depends on many factors, such as the physiological functions and conditions of the organ [8, 14, 16, 31, 34]. An increase in the DNA concentration is generally the most certain indicator of growth. The DNA structure is stable, e.g. P^{32} uptake occurs in DNA only when there exists mitotic activity and the amount of metabolized P^{32} is directly proportional to the mitotic activity [23, 24].

In DNA of the orbital gland, a significant increase due to the influence of orbital homogenate was found after 1, 2 and 7 days in both experiments. When evaluating the result it has to be kept in mind that the orbital gland grows very rapidly at this stage so that the influence on the 7th day or 10 days from the original value remains questionable. On the other hand, the short-time influences are obviously caused by the injected homogenate and seem to be due to growth stimulation.

The liver and orbital homogenates did not cause the increase of DNA in the liver. This only occurred in one case with the regenerating liver homogenate, in spite of a highly significant increase in RNA caused by the regenerating liver. The normal liver of new-born and young rats generally caused no changes in the liver.

When studying the nucleic acid concentration of the regenerating liver it has been found that the synthesis of DNA does not occur within 18 hours after partial hepatectomy. About 27 hours after the operation, a high rate of mitosis occurs, which is preceded by a rapid uptake of P^{32} into DNA. The maximal values in the DNA synthesis occur 24–30 hours after the hepatectomy, when its synthesis rate may be even ten times greater than that of the normal liver. The radioactivity level of RNA, on the other hand, is rapidly doubled in the first hours after hepatectomy [4, 13, 25]. In the opinion of the workers just cited, the synthesis rate of DNA has notably decreased after two days. It has also been reported that the RNA concentration per weight unit is at its highest 1½–6 days after partial hepatectomy and that the DNA concentration per weight unit reaches its maximal values 4–6 days after the operation [17].

The influence of the tissue homogenate injections is not, however, directly proportional to the changes caused by partial hepatectomy. In the latter case there is in question the increase of the nucleic acid synthesis caused by a real need, which does not apply in connection with the changes caused by injections.

According to our previous theory, there could exist in the tissues and organs an autoregulation of growth through the action of local intracellular stimulators and inhibitors [27, 28]. A stage of equilibrium could be prevailing under normal conditions, which is furthermore influenced by a superior hormonal pool. One of the best arguments for this theory so far is the mitosis stimulation of the injected organ homogenates. It has been presumed [29] that this might take place through the nucleic acids. The present work argues in favor of this theory.

SUMMARY

1. The influence of intraperitoneally injected homogenates of the outer orbital gland and liver from young rats and of regenerating liver on the nucleic acid concentration of these organs has been studied.

2. The influence of the orbital homogenate was specific, causing a significant increase in RNA and DNA of the orbital gland. It showed no influence on RNA and DNA of the liver. The influence of the homogenate prepared from the liver of new-born rats was not specific: it caused an increase in the DNA concentration of the outer orbital gland, and its influence on the liver was questionable; an increase in RNA was noted in one case only. The homogenate from the liver of 6-week-old rats had no influence on the liver; its influence on the orbital gland was not studied. The homogenate from the regenerating liver had no influence on the outer orbital gland, while RNA of the liver showed a significant increase in all experiments. The DNA had increased in one experiment only.

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BRIEF NOTES

OBSERVATIONS BY POLARIZED LIGHT ON THE SUPPORTING STROMA OF SOME ORGANS IN INSECTS

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In previously published investigations [1, 2, 3], carried out with ordinary histological techniques, supporting tissues had been found to occur in insects which exhibit morphological characteristics fairly similar to those demonstrated in parenchymatous stromas in vertebrates. Lamellar structures of the type of basement membranes were especially observed. The occurrence of filamentous structures evidenced by silver impregnation and the paraldehyde-fuchsin technique was particularly striking. These methods are, however, insufficient when it comes to solving the problem whether such filamentous structures are of the same nature as the collagen strands in vertebrates. Further chemical as well as submicroscopic investigations are required in order to elucidate this problem.

The present findings are based on preliminary researches carried out in polarized light. This method, accompanied by adequate topochemical tests, has so far supplied important data with regard to the identification of the nature of protein filamentous structures.

For the measurement of retardation values we used an Ortholux (Leitz) apparatus with built-in illuminating system, furnished with the compensators of Brace and Berek. The data obtained were checked on the apparatus belonging to the Institute of Anatomy in Milan (Ortholux (Leitz)) with mercury lamp and various types of compensators).

The basement membrane of the epidermis, intestine, and malpighian tubules, the lamellae and fine bundles which constitute the peri-intestinal connective tissue, the tunic of fat bodies, and the nervous sheath were studied in histological sections of *Anacridium aegyptium* L. (Orthoptera). When observed in distilled water all these structures show a birefringence which is positive with respect both to the long axis in the lamellar and fascicular structures and the tangent in structures of a basement membrane type. The retardation values range about 15/16 Å. Differences among the various structures have no significant value, as they may be due to slight differences in thickness.

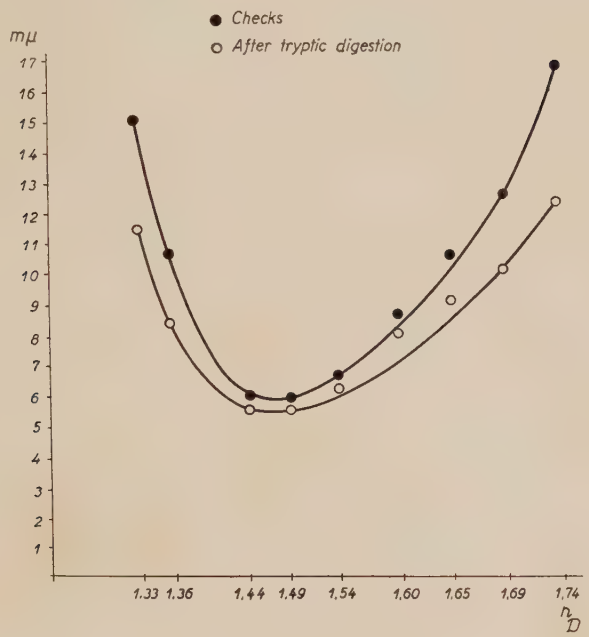

Fig. 1.—Curve of retardation values. *Abscissa:* refractive index of the imbibition liquid. *Ordinate:* retardation in Å units.

Characters of the Birefringence

Intrinsic and textural birefringence.—The tissue was imbibed with different fluids of progressively increasing refraction values, from distilled water (refraction index 1.33) up to methylene iodide (1.74). The birefingence of these structures appears to be partially related to the refraction indices: the retardation values decrease as the refraction index increases up to 1.49 where it reaches a minimum. Thereafter the values increase again so as to reach values which are similar to those in water.

This test reveals the double nature of the total birefringence: one is an intrinsic birefringence which does not change with the refraction index, the other is a textural birefringence, associated with the submicroscopic organization of the materials which build up a complex body according to Wiener.

The textural birefringence is of the same positive character as the intrinsic birefringence. Therefore, the existence is postulated of a filamentous complete body formed by submicroscopic threads embedded in substances of different refraction indices.

Enzymatic tests. –It has been demonstrated that structures under examination lose their birefringence and are finally melted by pepsin treatment at pH 2.5. They are restistant to trypsin in solution at a neutral or slightly alkaline pH. It must be stressed that under these conditions many structures showing a homogeneous lamellar appearance get a more definite filamentous aspect. In the residual threads the above mentioned characteristics of the birefringence are most evident. It might thus be demonstrated that trypsin is capable of removing the interstitial substance, but does not attack the filamentous structures.

Topochemical tests.—Osmic treatment which blocks the possible birefringence of lipids has given negative results. Similarly, prolonged lipid extraction does not alter the birefringence, thus allowing the conclusion that birefringence is not due to lipid structures.

Some alterations are brought about by acids and alkalies: this protein material does not exhibit the resistance to alkalies shown by keratins and chitins.

Phenol test.—By means of appropriate devices we have demonstrated that the birefringence is constantly reversed upon treatment with a concentrated alcoholic phenol solution and with aldehydes of clove oil. The index which was at first parallel to the filamentous structures is oriented orthogonally to the long axis and the tangent. This test is of great diagnostic value, since it always occurs in the structures of collagen-type. Picric acid did not yield any results; this may happen also with typically collagenous structures.

Conclusions

The present preliminary investigations confirm the occurrence in some supporting stromas of insects of filamentous structures, scarcely evidenced by transmitted light or phase contrast, which are near to the type of collagenous proteins of mammals, owing to their optical, enzymatic, and chemical properties. Such properties are: (1) positive intrinsic birefringence with respect to the long axis, (2) protein nature, (3) resistance to alkaline trypsin, (4) weak resistance to alkalies and acids, (5) positive test of phenolic inversion, and (6) resistance to lipo-solvent substances.

We cannot state whether these structures possess the submicroscopic characteristics of the collagen of mammals (submicroscopic filaments with a periodic structure of 640 Å), or are nearer to some organizations of collagen-type such as byssokeratin, spongin, or some cuticular structures in worms [4].

The characteristics of the textural birefringence seem to indicate the existence of submicroscopic threads embedded in a substance of different refraction index. This substance may be removed through tryptic digestion, and shows a positive response to the following histochemical tests for polymers of carbohydrates: PAS reaction, PAS reaction after deacetylation, PAS reaction after ribonuclease controls, PAS reaction after hyaluronidase treatment (PAS after acetylation and after the blocking of aldehydes by bisulphite shows a negative response), toluidine blue method (standard), dialysed iron method for acid mucopolysaccharides.

In further investigations, particularly by means of the electron microscope, it may become possible to show how far these stromas are comparable to the connective tissues in mammals.

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IMPROVED FIXATIVE FOR ASTRAL RAYS AND NUCLEAR MEMBRANE OF *TUBIFEX* EMBRYOS

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Previous studies on *Amoeba* and *Tubifex* have shown that the preservation of fibrous cytoplasmic structures is not very satisfactory after treatment with neutral osmic fixatives, e.g. fixative of Palade [9] or fixative of Weber [10]. A suitable rapidly penetrating and partly dehydrating fixative was found for *Ilyanassa* eggs [7] and adapted for *Tubifex* eggs. This "fixative D" contains one part of solution I (80 ml of 20 per cent formaldehyde, 16 ml of acetone and 0.5 ml of acetic acid [7]) to one part of solution II (2.5 per cent osmium tetroxide). Embryos were placed in this fixative for 30 minutes. A still better preservation of some lipid and also some fibrous structures was obtained by a subsequent treatment with chromic acid. Two milliliters of 10 per cent chromic acid were added for every 5 ml of fixative D. The chromic acid was added 20 minutes after the embryos had been placed in fixative D. After 20 minutes of exposure to this combination (fixative E) the eggs were rinsed for 15 minutes in tap water. They were then ready for dehydration and embedding in butyl–methylmethacrylate (9:1), which was done in the usual way. We studied 0.05–0.1 μ thick sections made with a Porter–Blum microtome.

The sections, either with or without previous removal of the methacrylate, were photographed with a Trüb–Täuber electron microscope. The dissolving of the polymerized methacrylate by amylacetate produced considerable distortion of fine structures. In contrast to this a mixture of unpolymerized butyl–methylmethacrylate (9:1) dissolves the polymer without noticeable damage to cellular structures. Therefore, this procedure was adopted finally for routine work.

Structure of aster and nuclear membrane.—(1) Cleavage asters: The asters in many species with spiral cleavage are large structures visible either in living cells (*Ilyanassa*) or in fixed and sectioned embryos (*Tubifex* [6]). These fibrous structures should be visible in detail on electron micrographs, provided a suitable fixative has adequately preserved them. Our new fixatives D and E have given many clear pictures of the astral area of *Tubifex*, which is situated close to the interphase nucleus. The astral center consists of a very dense sponge-like reticulum (see Beams *et al.* [2, 3]) which contains no large bodies such as mitochondria or lipid droplets. A centriol-like structure, as recently described by de Harven and Bernhard [4] for vertebrate cells, has not yet been found in *Tubifex*. The fibers of the astral reticulum are similar in size to those of the endoplasmic reticulum of *Amoeba*. They contain weakly electron-absorbing fibrous material and very dense microsome-like bodies. The more distal part of the aster possesses a great number of very long straight fibers

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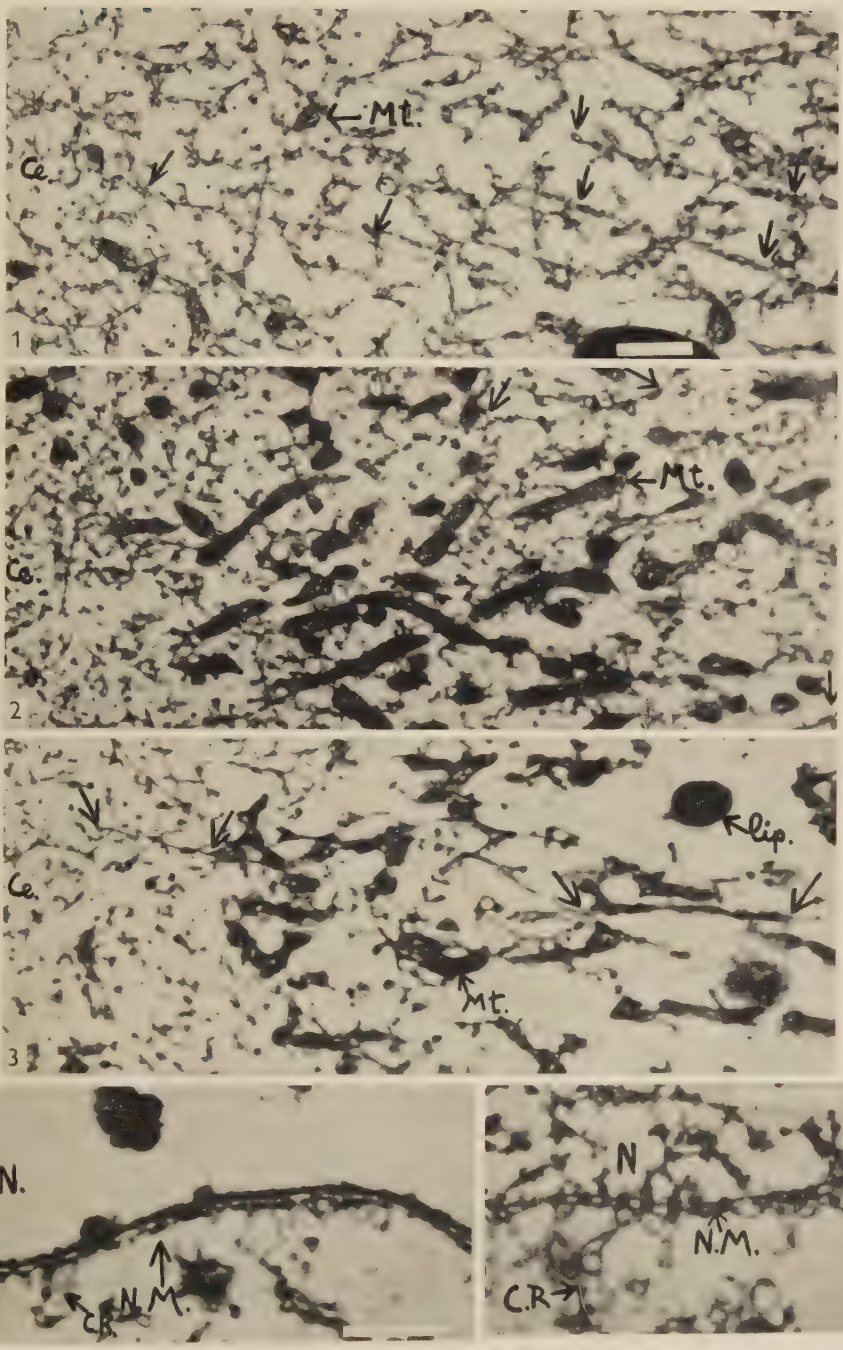
running mostly in a radial, rarely tangential direction. The asters of the three main cells, namely, the ectoblast 2d, the mesoblast 4d and the entoblast 4D show characteristic differences in structure and composition probably caused by some intrinsic differences in the cytoplasmic architecture of the respective cells. The aster of 2d (Fig. 1) contains in the central part of the aster a dense network of fibers, the diameter of which averages about 30 mμ. In the peripheral part numerous straight fibers are found, predominantly in a radial arrangement. A few tangential fibers are also present. The length of the fibers in our micrographs reaches 3000–4000 mμ, their diameter 30–80 mμ. These fibers are nonhomogeneous, containing along their fibrous axis microsome-like bodies and larger "osmiophilic vesicles" [10]. There are also present some mitochondria which seem to be less numerous in this aster than in that of 4d.

The aster of 4d (Fig. 2) differs considerably from that of 2d in exhibiting a higher density of the fibrous reticulum and a greater number of mitochondria. Also in 4d the central network of the aster is free from larger bodies (mitochondria, etc.). In the peripheral astral zone large mitochondria 2000–5000 mμ in length and straight astral fibers are present as in 2d. The large entoblast 4D (Fig. 3) is rather poor in cytoplasm and the same holds also for its aster. The central reticulum of the aster is loose and contains fewer fibers than those of 2d or 4d. In the peripheral part some astral fibers have a diameter as in 2d of about 30 mμ, others are very large with diameters of about 100 mμ. These fibers seem to be fused with some peculiar mitochondria-like structures. Other mitochondria are only loosely attached to the astral reticulum. These structural features distinguish clearly the aster of 4D from the asters of 2d and 4d.

Thus far the astral structures described here have been found only in the cells of several embryos fixed either with fixative D or E. The astral structures of the embryonic cells of *Tubifex* prepared with our methods resemble more closely the living or isolated structures (mitotic apparatus of the sea urchin, [8]) or the usual (Carnoy) cytological preparations, than the ones which until now have been preserved by osmic fixatives (see, for instance, de Harven and Bernhard [4]). They are, on the other hand, very similar to those shown by Beams *et al.* [2, 3], who fixed the asters with Bouin's fixative. Bouin's fixative is known to preserve fibrous structures rather well [1].

All figures are electron micrographs of ultrathin sections of *Tubifex* blastomeres from a 21-cell stage. Abbreviations: *Ce*, central part of the aster; *Lip*, lipid droplet; *Mt*, mitochondria. The arrows indicate the extent of some astral fibers. The white strips correspond to one micron. All blastomeres were fixed in fixative D.

Fig. 1.—Aster of the ectoblast 2d.
Fig. 2.—Aster of the mesoblast 4d.
Fig. 3.—Aster of the entoblast 4D.
Figs. 4 and 5.—Electron micrographs of nuclear membranes of *Tubifex* blastomeres. *Fig. 4.* Nuclear membrane of the entoblast 4D. The nuclear space (*N*) appears empty, except for a few large chromatic bodies. The inner part of the nuclear membrane is rather dense and relatively thick. The outer layer is thinner and contains globular bodies connected with the inner part. *C.R.*, cytoplasmic reticulum of the adjacent cytoplasm. *Fig. 5.* Nuclear membrane of the blastomere 2d. The nuclear space (*N*) contains a reticulum. Both inner and outer parts of the nuclear membrane are thin. The two layers are connected by globular bodies. The cytoplasmic reticulum (*C.R.*) (below) is rich in vesicular bodies.


Mt.
Ce.
1
Mt.
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2
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N
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C.R.
5

(2) Nuclear membranes. A large series of observations on very different animals indicates a wide occurrence of two-layered nuclear membranes. The embryonic nuclei of *Tubifex* also possess this type of membrane [10]. The fine structure of these membranes is well preserved by the new fixatives D and E. There are some differences between the nuclear membranes of 2d and 4d, on the one hand, and of 4D on the other. Between the outer and the inner nuclear membranes of 2d and 4d there occur characteristic globular bodies (Fig. 5). These bodies seem to hold the two membranes together. They contain a central cavity, clearly visible in tangential sections. The diameter of the central part is about 30 mμ.

The membrane of the entoblast nucleus 4D (Fig. 4) shows some conspicuous differences. The inner membrane is here much thicker, about 120–160 mμ, and is covered by a thinner outer layer which is about 80 120 mμ in diameter. The two layers are partly separated by a more transparent layer; yet they are partly connected by strongly electron-absorbing globules or spheres the outer diameter of which is about 200 mμ. The diameter of the light area of the spheres is about 30 mμ.

The general appearance of the nuclear membranes of the blastomeres of *Tubifex* is similiar to that of other nuclear membranes, to judge from the recent description by Haguenau and Bernhard [5] (see Fig. 6). Therefore, we may assume that our new technical procedures produce electron micrographs of satisfactory equivalence to the living state. These methods seem to be superior, at least for our material (*Amoeba* [1] and *Tubifex*), to the usual osmic fixatives, because they preserve fibrous structures very well. Moreover, we did not observe cloudy and partly disintegrated structures commonly seen in electron micrographs of tissues fixed with the osmic fixatives. A comparative study of the new and old fixatives as applied to the embryos of *Tubifex* is in preparation.

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ELEKTRONENMIKROSKOPISCHE BEOBACHTUNGEN ÜBER DAS VORKOMMEN VON PLASMODESMEN IN ZELLWÄNDEN[1]

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Das Vorhandensein von Plasmodesmen in den Zellwänden konnte in der letzten Zeit mehrfach nachgewiesen werden. Lambertz [3] gelang es lichtoptisch, sehr überzeugend bei zahlreichen Pflanzenarten Plasmodesmen in den Epidermisaussenwänden darzustellen; Huber, Kinder, Obermüller und Ziegenspeck [2] zeigten bei *Helleborus* elektronenoptisch derartige Plasmodesmen in den Schliesszellen von Spaltöffnungen sowie in den Epidermisaussenwänden; Strugger [5] fand eine grosse Zahl dieser Zellverbindungen in den meristematischen Zellen der Wurzelspitze von *Allium cepa*. Bei unseren elektronenmikroskopischen Untersuchungen konnten wir ähnliche Strukturen in den Wänden der Pollenmutterzellen von *Gasteria trigona* und *Lilium candidum*, sowie in den verholzten Tracheidenwänden von *Pinus silvestris* beobachten.

In den Wänden von Pollenmutterzellen der späten Prophase und der Metaphase der Meiosis sind sowohl nach Fixierung mit dem Flemming-Heitz-Gemisch als auch nach Osmiumsäurefixierung zahlreiche stark osmierte Poren oder Stege zu erkennen (Abb. 1). In diesem Stadium befinden sich die Zellen nicht mehr im Gewebeverband, sondern liegen voneinander isoliert. Sie zeigen zudem eine mehr ovale Form, wobei die Zellwände an den Polen verstärkt ausgebildet sind. Vorzugsweise an diesen Polen sind die dunklen Poren in grösserer Zahl zu finden. Sie haben meist einen ziemlich regelmässigen Abstand und zeigen eine vorwiegend radiale Orientierung. Bisweilen können sie sich sowohl zentripetal als auch zentrifugal gabeln. An den jeweiligen Enden sind sie meist leicht trichterförmig verbreitert. Ihr Durchmesser beträgt etwa 110–200 mμ.

Die Interpretation derartiger Strukturen bedarf besonderer Vorsicht, da sehr ähnliche Gebilde auch durch Falten oder durch Stauchungen des Einbettungsmittels entstehen können. Auch Huber und Mitarbeiter weisen daher auf diese Gefahr besonders hin. Gegen die Annahme von Artefakten spricht aber, dass die Poren stets streng auf die Zellwand begrenzt sind und dass sie mitunter gebogen verlaufen, winklig zueinander stehen oder auch nur angeschnitten erscheinen können. Da sie zudem sehr regelmässig und gleichförmig auftreten, glauben wir, dass sie realen Strukturen entsprechen. Dennoch können wir die Möglichkeit von Artefakten nicht vollständig ausschliessen, zumal uns Herr Dr. Bahr kürzlich freundlicherweise Aufnahmen mit ganz ähnlichen Bildungen zusandte, die von einem Objekt stammen, das keine Plasmodesmen besitzt.

Daneben fanden wir aber auch in den verholzten Zellwänden der Tracheiden von verschiedenen Gymnospermenarten vereinzelt kleine Öffnungen von etwa 1 μ

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft.

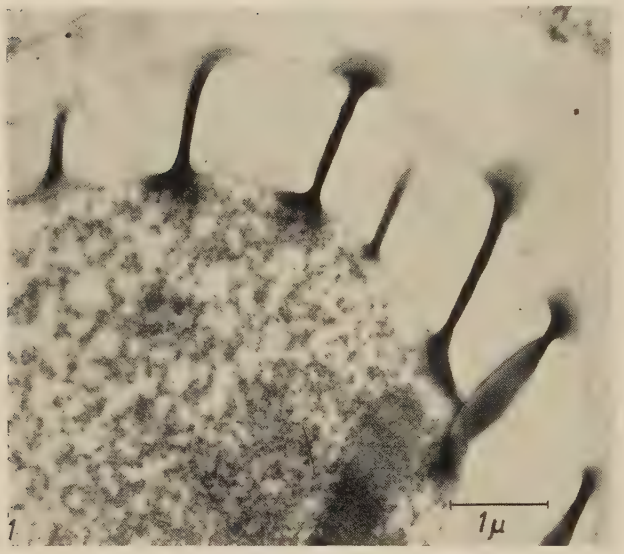

Abb. 1.—Pollenmutterzelle von *Gasteria*, Metaphase; verdickte Zellwand mit zahlreichen Plasmodesmen; 15000×.

Abb. 2.—Sekundärwand einer Tracheide von *Pinus silvestris* im Radialschnitt mit zwei Hoftüpfeln und einer Plasmodesmenpore; 4100×.

Grösse, die durch ein stromlinienförmiges Umfliessen der Elementarfibrillen ausgespart werden (Abb. 2). Diese Poren konnten jedoch nur in der Sekundärwand, nicht dagegen in der darüberliegenden und die Tracheide gegen das Lumen abgrenzenden Tertiärwand festgestellt werden. Es ist denkbar, dass diese Öffnungen während der Zelldifferenzierung als Plasmodesmen dienen. Auffallend ist, dass sie sogar in unmittelbarer Nähe von Hoftüpfeln vorhanden sein können, durch die ja bereits eine intensive Plasmaverbindung während des Wachstums gegeben sein dürfte. Das Fehlen der Poren in der Tertiärwand unterstreicht die Auffassung, dass die Tertiärwand ontogenetisch nach der Sekundärwand entsteht [1, 4]. Da diese Beobachtungen mit einem Polymerisationsabdruckverfahren gemacht wurden, ist die Gefahr von Präparationsartefakten recht gering. Auch zeigt die Textur der Elementarfibrillen, dass die Poren nicht durch das Wachstum von Pilzhyphen durch die Zellwand entstanden sein können.

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DNA SYNTHESIS IN TWO CILIATES

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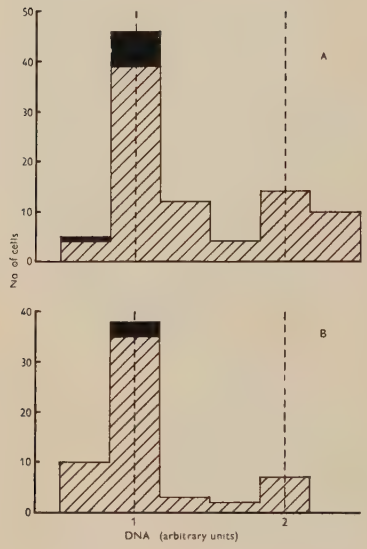
Measurements on the Feulgen stained nuclei of growing and dividing cells have made it feasible to calculate the rate and time of DNA synthesis during the life cycle of an average cell. So far this method has only been used on the cells of higher animals [5, 9], so we decided to extend it to Protozoa.

The amount of Feulgen stain present has been measured by a scanning instrument devised by Deeley [2], which by eliminating errors due to the inhomogeneity of the stain can give very accurate results. Measurements from a large number of cells can be plotted as a frequency histogram, the shape of which is in general determined by the shape of the synthesis curve of DNA. The method used to obtain such a synthesis curve has been already described [7] and reasonably good agreement is found between these synthesis curves and those obtained by direct measurement and observation of tissue culture cells [5, 9].

For this calculation it is necessary to assume that (1) population growth is logarithmic, (2) the DNA doubles during a complete cell cycle (3) there is little or no



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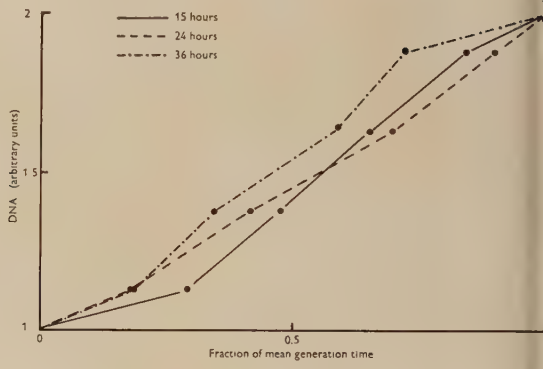

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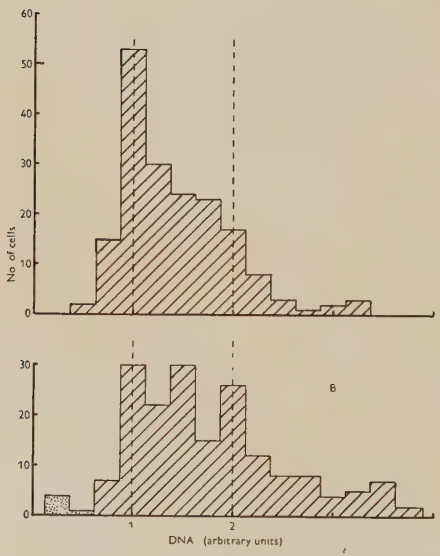

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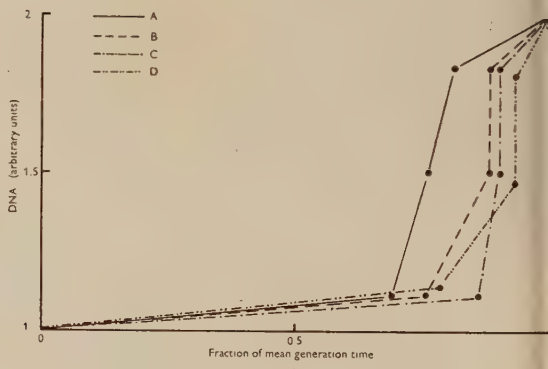

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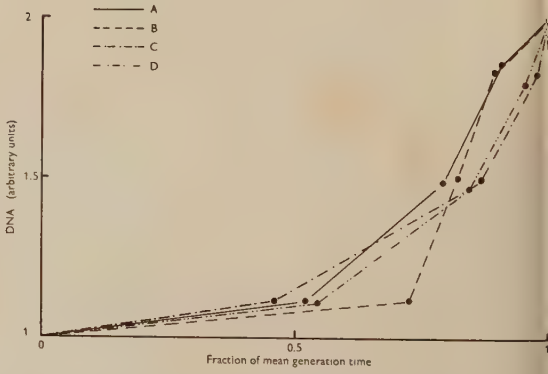

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Fig. 1.—Macronuclei of *Tetrahymena pyriformis* sampled (*A*) 15 hours—181 cells—and (*B*) 36 hours—186 cells—after inoculation. The stippled blocks represent nuclear buds.

Fig. 2.—Synthesis curves calculated from the distribution of cells within the one to two unit blocks of the histograms from *Tetrahymena pyriformis*. The 36-hour curve is included for comparison only.

Fig. 3.—Representative histograms from (*A*) macronuclei and (*B*) micronuclei of *Paramecium caudatum*. The filled blocks represent recently divided cells.

ERRATA

P. M. B. Walker and J. M. Mitchison, "DNA synthesis in two ciliates", this journal **13**, 167:

Page 168: *The top-left figure is Fig. 3, and the bottom-left figure is Fig. 1.*

169, line 6: *For* a micro-nucleate *read* an amicro-nucleate

synchrony of division and (4) the variation in time and rate of synthesis between different cells is not so large as to reduce the significance of an *average* synthesis curve.

There is a lower limit to the sensitivity of the instrument and therefore many of the smaller organisms cannot be measured in this way. We have confined our measurements to (*a*) a micro-nucleate strain of *Tetrahymena pyriformis* and (*b*) the micro- and macro-nuclei of *Paramecium caudatum*. We tried to use *P. aurelia* but its micronucleus was too small for accurate measurement.

Tetrahymena pyriformis. Strain W.—These were grown in 2 per cent peptone at 27°C, and under these conditions had a minimum mean generation time of 3½–4 hours. The cultures were sampled at 15 and 24 hours after inoculation, during the logarithmic phase and at 36 hours when growth was slower. Each sample was concentrated by centrifuging before being lightly smeared onto a coverslip. The cells were air dried, fixed in acetic alcohol and Feulgen stained [8].

It was found that the histograms from the two younger samples were similar (one of these is shown in Fig. 1 *A*), and their shape indicated that in the macronucleus the DNA doubles from one to two arbitrary units for most of the population. On the other hand the histogram of the 36 hour sample had a different form (Fig. 1 *B*), in which about 30 nuclei had DNA values well above the 2 unit value and in which there was some evidence of an intermediate peak at 1.5 units. This sample also included a number of the nuclear buds described by Furgason [3], which were not seen in the younger samples. This suggests that in some of the cells in the older culture DNA synthesis continues after the 2 unit value has been reached up to the time when division occurs. These cells then divide to give the peak about 15 units. If this is so, nuclear budding might be a mechanism for adjusting the amount of DNA to the more normal one unit value.

Since in the younger samples there were no buds and fewer cells outside the one to two unit range, it seemed justifiable to assume that the DNA in most cells doubled in the usual fashion and that an average synthesis curve can be obtained. This would not be justified if most of the intermediate cells had resulted from the division of the small number of high valued (> 2 units) cells measured. Synthesis curves for the 15 and 24 hour samples are shown in Fig. 2, and they appear to be roughly linear throughout the interphase period of about four hours. For comparison the curve calculated from the cells within the one to two unit blocks of the 36 hour sample is also included.

This linear synthesis curve is different from those obtained from cells grown in tissue culture, which show evidence of an initial lag period, and it seemed possible that the synthesis curves of macro- and micro-nuclei might be different. We therefore decided to make similar measurements on *Paramecium caudatum* which has both kinds of nuclei.

Paramecium caudatum.—These were grown at 27°C on *Aerobacter* in baked lettuce

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Fig. 4.—Synthesis curves for micronuclei of *Paramecium caudatum*. *A*, 50 cells acetic alcohol-fixed near end of lag phase of growth; *B*, 25 cells formalin-fixed as those of *A*; *C*, 58 cells acetic alcohol-fixed as those of *A*; *D*, 44 cells formalin-fixed in logarithmic phase of growth.

Fig. 5.—Synthesis curves for macronuclei of *Paramecium caudatum*. *A*, 76 cells formalin-fixed near end of logarithmic phase of growth; *B*, 80 cells like those of *A*; *C*, 90 cells formalin-fixed in logarithmic phase of growth; *D*, 85 cells like those of *C*.

medium and had a minimum mean generation time of 10 hours. The cultures were concentrated by filtering through a filter candle and centrifuging. The cells were then air dried onto a coverslip, fixed in either acetic alcohol or formalin vapour and Feulgen stained. The method of fixing did not affect the shape of the histograms.

The macro-nuclei of *P. caudatum* stain very intensely; they were therefore flattened by the crushing method [1] and the hydrolysis times were reduced to decrease the amount of stain. Measurements on other cell types have indicated that such a reduction does not alter the shape of the histogram in a population of dividing cells; in other particulars the normal staining procedure was used. The micronuclei were often overlain by or embedded in the macronuclei and if this happens only at a particular stage during interphase, sampling errors will result.

In the first experiments (Fig. 4 *A*, *B*, *C* and 5 *A*, *B*) the sample was probably taken during the logarithmic phase of growth, but rather near to the beginning of the stationary phase. In the second set (Figs. 4 *D* and 5 *C*, *D*) the samples were definitely in the logarithmic phase. Typical histograms are shown in Fig. 3 *A* and *B* for macro- and micro-nuclei respectively, and it will be seen that there are far fewer cells with intermediate quantities of DNA than with *Tetrahymena*. This difference is reflected in the synthesis curves illustrated in Figs. 4 and 5, which shows a considerable lag period in both types of nuclei before DNA synthesis begins. The differences in the length of the lag period are probably insignificant in view of the possible errors in measurement and calculation.

The relative amounts of DNA in macro- and micro-nuclei are also of interest. The instrument was calibrated by measuring ram sperm under the two conditions of magnification and sensitivity required for the two types and it was found that in the cells measured there was about 25 to 30 times as much DNA in the macro-nucleus as in the micro-nucleus. This is similar to the ratio found by Moses [4] who used a less direct method of measurement.

The DNA synthesis curves for both the macro- and micronuclei of *Paramecium* are similar to those found for the nuclei of other animal cells [5, 8]: the DNA doubles fairly quickly towards the end of interphase. At first sight, however, the linear synthesis of DNA in the *Tetrahymena* macronuclei is surprising. But it may well be that this is due to the much shorter generation time in *Tetrahymena* since the actual duration of the synthesis is much the same in both animals. There is in fact evidence that tissue culture cells with a long generation time take about the same time to double their nuclear DNA as cells with a short generation time and it is the initial lag period before synthesis which is greater [5].

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THE ABSENCE OF α-ε-DIAMINOPIMELIC ACID FROM THE PRIMITIVE RED ALGA *PORPHYRIDIUM CRUENTUM*[1]

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α-ε-Diaminopimelic acid (DAP) has been shown to be characteristically present in most bacteria and the few blue-green algae so far studied [11, 4] and, in general, to be absent from higher organisms [11]. Where it occurs naturally, it is in the form of the *meso* isomer as a rule, although the LL-isomer is characteristic of some bacteria (e.g. certain actinomycetes [7]). It is known to have several functions in bacteria, among them being to serve as a constituent of cell wall polypeptides in certain forms [9]; but its role in the blue-green algae is unknown [4]. The fact that DAP is not restricted to the Monera (bacteria + blue-green algae) has recently been established, with the discovery of small traces (isomer undetermined) in the green alga *Chlorella ellipsoidea* [5, 10]. In general, however, it can be said that relatively high levels of DAP are found only in the Monera, of which only a few forms (Gram-positive cocci) have been found to lack it [11, 7].

The genus *Porphyridium* Nägeli, 1849, and allied genera of the order Porphyridiales comprise species of unicellular red algae regarded by many as very primitive members of their phylum (Rhodophyta), possibly intermediate between the blue-green algae (phylum Archephyta, class Cyanophyceae) and the higher rhodophytes [3]. Because of the apparent importance of DAP as a phylogenetic marker, we feel that a report of recent testing of *Porphyridium cruentum* (C. Agardh) Nägeli is of particular interest.

P. cruentum (Vischer strain) was grown in axenic culture in a synthetic sea water medium [1]. 250 ml portions were placed in Roux bottles and shaken over a bank of fluorescent lights delivering ca. 10,000 lux at the level of the bottle. Five per cent CO_2 in air was supplied to the cultures. After 8–10 days' growth the cells were harvested by centrifuging, washed with water, extracted with 80 per cent acetone, and dried *in vacuo* at room temperature.

180 mg of the defatted cells were hydrolyzed with 6 *N* HCl as described by Work and Dewey ([11], p. 395), and the hydrolysate evaporated to dryness. The dry film was redissolved in 1.1 ml water.

The resulting solution was painted with a camel's hair brush along a line, 49 cm long, drawn parallel to, and 4 cm from, the longer edge of a 24 × 57 cm sheet of Whitman No. 4 filter paper. The sheet was rolled into a cylinder with the line of hydrolysate parallel to one end, and the cylinder was then stood on that end in a

[1] Contribution no. 2 from the Laboratory of Comparative Physiology and Morphology of the Kaiser Foundation.

closed vessel containing, at the bottom, a few mm of pyridine–isopropanol–water–acetic acid (8:8:4:1), a solvent mixture [6] of which we can here report for the first time that DAP has a smaller R_F in it than have any of amino acids of casein hydrolysate. After this solvent had ascended for 2 hours, the paper was removed from the solvent and dried, and the strip containing DAP cut and eluted by an ascending front of water. The first few lambdas of aqueous eluate were spotted and chromatographed in methanol–water–10 *N* HCl–pyridine (8:1.75:0.25:1) as described by Rhuland *et al.* [8] for the separation and detection of DAP.

Parallel chromatograms were run on dried cells of the blue-green alga *Anabaena cylindrica* Lemmerman, which by previous report [11] contains DAP. These cells were grown as described previously [2] and dried *in vacuo* without defatting.

No olive-green spot appeared in the calculated area in the case of *P. cruentum*, whereas, in the case of *A. cylindrica*, a large spot appeared which changed to a permanent yellow and was deeply fluorescent under u.v. light. Despite the initial separation of amino acids, there was still a considerable amount of streaking in the presumptive or actual area of DAP in each case, because of heavy overloading of the spot on the final chromatogram, but all other colors ultimately faded, an essentially colorless strip being left in the case of *P. cruentum*.

In this system, 10 γ of DAP could have been readily detected. Therefore if any DAP is present in *P. cruentum*, it occurs at a level less than 0.005 per cent of the dry weight (after defatting). We must thus conclude that, according to the criteria used by Work and Dewey [11], DAP is lacking from *P. cruentum*.

This absence need not be taken to mean that *P. cruentum* must be dismissed as representing a group intermediate between the Cyanophyceae and Rhodophyta, but does support its placement in the latter as opposed to the former taxonomic assemblage. Many provocative questions are thereby raised. Since it is essentially the segregation of the nuclear and photosynthetic apparatus into intracellular compartments that sets the unicellular red algae off morphologically from the blue-green algae, one may well ask: is the presence of DAP related to certain features of cellular architecture (primitve nucleus [all monerans]; dispersed grana [photosynthetic monerans]) characteristic of the Monera; and, conversely, does organization of nuclear material and of grana into special compartments within the cell do away somehow with the need for high levels of DAP? Answers to these questions may go a long way not only toward explaining the basic role of DAP in the Monera, but also toward elucidating the biochemical consequences of the transformation that occurred when monerans evolved into intermediate (red-algal) and higher protists.

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CATION BINDING AND EXCHANGE IN KILLED RED ALGAL TISSUES

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During ion transport studies on the red marine alga, *Porphyra perforata* J. G. Agardh, the binding of rather large amounts of sodium and calcium was observed in tissues killed by brief immersion in boiling water or by oven drying at 100°C. Boiled pieces of the alga dipped in sea water, then washed in 0.6 *M* sucrose, blotted with tissue paper, and weighed, contained 40 milliequivalents per kilogram fresh weight (mE/kg FW) of sodium and 10 mE/kg FW of calcium.

Cation binding has been previously reported for brown [3], green [11], and blue green algae [9], as well as for purified algal polysaccharides such as alginic acid from brown algae [7], and agar [2], usually obtained from the red alga, *Gelidium* sp. Sulfonated galactans such as agar, but not alginic acid, are common in red algae [1], probably occurring as cell wall constituents [4].

Sodium bound by killed *Porphyra* tissues may be replaced by other cations on immersion of the sodium-charged alga in dilute acid or salt solutions. The amount of replacement was determined by measuring the sodium bound before and after exposure to the respective solutions (Table I). Trivalent aluminum replaced the most sodium, followed by calcium and magnesium, rubidium, potassium, and lithium. Such an order might be expected if the killed *Porphyra* thallus was acting as a cation exchange material [12]. This is in marked contrast to living cells of *Porphyra* which exclude sodium and accumulate potassium [3].

A polysaccharide capable of binding cations was isolated from *Porphyra* by boiling plants 23 hours at pH 5, filtering, dialyzing overnight, and precipitating with ethanol. The resulting material, further purified by dissolving in cold water and reprecipitating with ethanol several times, moved as one spot on paper chromatograms in ethyl acetate–pyridine water (2:1:2, v/v) and *n*-butanol acetic acid–water (4:1:5, v/v) solvent systems. Hydrolyzed material showed spots on the paper chromatograms identical with galactose and xylose after development with the above solvent systems and spraying with aniline phthalate [8]. Uronic acid did not appear on the chromatograms but its presence was indicated by a colorimetric test [10].

[1] Public Health Service Pre-doctoral Fellow of the National Heart Institute.

The hexose content of the polysaccharide was 62 per cent (as galactose [6]), pentose 11 per cent (as xylose [6]), uronic acid 4.9 per cent (as glucoronic acid [10]), sulfate 11.2 per cent (as SO_4 [5]) and sodium 7.8 per cent on a dry weight basis after exposure to 1 *M* NaCl and washing with ethanol. Similar polysaccharides have been reported for other red algae [1].

TABLE I

Exchange of cations for bound sodium in killed thalli of Porphyra perforata.

Algae dried at 100°C, ground and sieved to 24–48 mesh were used in Expt. 1. Algae killed by brief immersion in boiling water were used in Expt. 2. In each case the initial sodium values, determined by flame photometry, represent sodium bound by the algal tissues from sea water. The plant materials were soaked in the various solutions four or five days, washed free of adhering solution with distilled water, and the remaining sodium was determined.

Expt. No.	Solution	Sodium remaining mE/kg dry wt.	% sodium exchanged
1	0.1 *M* LiCl in 0.1 *M* NaCl	203	4.7
	0.1 *M* KCl in 0.1 *M* NaCl	165	22.5
	0.1 *M* RbCl in 0.1 *M* NaCl	93	56
	initial sodium value	213	0
		mE/kg fresh wt.	
2	0.1 *M* KCl	21.4	46
	0.1 *M* $Mg(acetate)_2$	5.8	86
	0.1 *M* $Ca(NO_3)_2$	6.1	85
	0.1 *M* $AlCl_3$	3.4	92
	initial sodium value	40	0

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LYSIS OF *BACILLUS SUBTILIS* BY CYSTAMINE[1]

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The ability of ethylene diamine tetraacetate (EDTA) to promote lysis of gram negative bacteria by lysozyme has been reported recently by Repaske [3] who noted also that cultures of *Pseudomonas aeruginosa* are lysed partially by EDTA itself. A search for other metal binding substances with bacteriolytic properties has revealed that cystamine[3] (NH_2–CH_2–CH_2–S–S–CH_2–CH_2–NH_2) causes cells of strains of *Bacillus subtilis* to lyse completely under the conditions described in this communication.

Cultures used in this preliminary study were obtained from the department stock culture collection. Several strains each of various species of Pseudomonadaceae, Enterobacteriaceae, Micrococcaceae, Mycobacteriaceae, and Bacillaceae were employed. Tests on solid media were performed with nutrient agar or a synthetic salts–glucose agar; autoclaved or filtered cystamine was incorporated in the media in the upper layer of double gradient plates [5]. Tests in liquid media were performed in nutrient broth contained in either shaken or stationary flasks. Inocula containing approximately 104 viable cells from 12-hour-shaken broth cultures were spread evenly over the agar surfaces or added to the individual flasks. All cultures were incubated at 37°C unless otherwise noted.

On nutrient agar gradient plates that contained either filtered or autoclaved cystamine (100 μg/ml) in the upper layer, complete growth of each of the test strains occurred. However, between 20 and 30 hours after inoculation, fading of the colonies of strains of *Bacillus subtilis* began in the half of the plate that contained > 50 μg/ml cystamine; by 72 hours, visible growth had disappeared. Strains of *B. cereus*, *B. anthracis*, and *B. brevis*, exhibited moderate to slight fading; no effect was obtained with strains of *B. megaterium*, *B. sphaericus*, or *B. polymyxa*. Strains of all other bacterial species tested were likewise resistant to cystamine lysis.

With *B. subtilis*, incorporation of metallic salts in the lower layer of nutrient agar double gradient plates revealed that Cu^{++} enhanced the action of cystamine; $Fe^{++}(^{+})$, Al^{+++}, Co^{++}, Ni^{++}, Zn^{++}, Mn^{++}, Mg^{++}, and Ca^{++} were inactive. Lysis could not be demonstrated on the synthetic salts–glucose agar with either cystamine or cystamine plus Cu^{++}. The final pH of cultures in the synthetic medium was 6.8 in contrast to a final pH of 8.5 in the complex nutrient medium.

In shaken nutrient broth containing 50 μg/ml cystamine, lysis of *B. subtilis* began after the completion of logarithmic growth and proceeded at the rate shown in Fig. 1. Control cultures in plain nutrient broth remained turbid for 5 days at which time

[1] Supported by a grant from the National Science Foundation.
[2] With the excellent technical assistance of Carolyn A. Wisner.
[3] Cystamine dihydrochloride (#9054 and #9810) purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

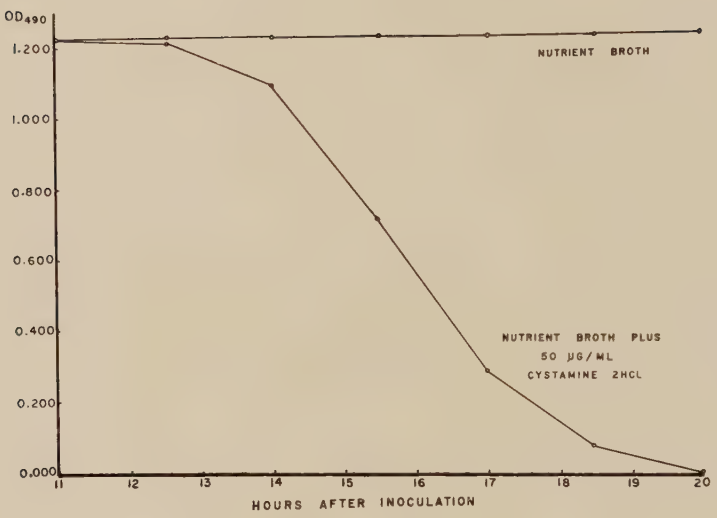

Fig. 1.—Lysis of Marburg strain of *B. subtilis* observed by following the optical density change at 490 mμ in a Beckman spectrophotometer.

they were discarded. When 50 μg/ml cystine was included with cystamine in nutrient broth, lysis by cystamine was delayed for 4–6 hours.

At 24 hours after inoculation, nutrient broth control flasks contained 10^8 viable cells/ml whereas cystamine lysed flasks contained less than 10^2 viable cells/ml. Serial subculture of cells from lysed flasks into fresh nutrient broth plus cystamine resulted in subsequent generations of cells that were neither more nor less resistant to lysis by cystamine. It has not yet been possible to demonstrate the presence of a lytic principle either in the cell-free filtrate from 24-hour control flasks or in the cell-free lysate from 24-hour lysed flasks.

Addition of cystamine to nutrient broth cultures at any time during the maximum stationary phase resulted in lysis after a subsequent period of 4–8 hours. When cystamine was added initially and the flasks were removed from the shaker at the end of logarithmic growth, lysis occurred as rapidly under stationary conditions as if shaking had been continued. Lysis also occurred when the flasks, at the end of logarithmic growth, were placed at 45°C or 55°C but not at 20°C or 8°C. Cells grown and maintained at 20°C in either nutrient broth or nutrient agar plus cystamine did not lyse. When cells grown and maintained in shaken cultures with cystamine at 37°C were heated at the end of logarithmic growth for 5 minutes at 60°C or 75°C and then were returned to 37°C, lysis proceeded as usual in the 60°C exposed cultures but did not occur in the cultures exposed to 75°C. When cells from plain nutrient broth at the end of logarithmic growth were washed and resuspended in 0.05 *M* phosphate buffer with and without cystamine, lysis occurred with cystamine in buffer adjusted to pH 8.0 but not pH 7.0.

As lysis proceeded on agar or in broth, the percentage of Gram negative cells in the population increased; with continued lysis the number of cells that could be seen by Gram stain gradually diminished and in completely lysed cultures, no cells could be observed. Likewise, the number of cell walls (observed with the acetyl

pyridinium-Congo Red stain) decreased during lysis and cell wall material could not be seen in completely lysed cultures.

In recent years, lytic phenomena in cells of the genus *Bacillus* have been reported with increasing frequency (summarized by Norris [2]). Of possible significance is the fact that often small sulfur containing and/or metal binding molecules such as H_2S, thioglycollate, merthiolate, glycine, and cystamine are implicated as triggering agents for lysis. The phenomenon reported in the present communication resembles the two stage lytic systems described by Stacey and Webb [4] and by Cowles [1] in which loss of Gram positiveness is followed by disintegration of the Gram negative cytoskeletons at alkaline but not acid pH reactions.

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THE EFFECT OF DEXTRAN ON THE DRY MASS DISTRIBUTION IN OSMOTIC HEMOLYSIS

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Marsden and Zade-Oppen [7] found that the osmotic resistance of human cells was considerably increased by the presence of dextran or human serum albumin in the suspending medium. From the appearance of such cells in a phase contrast microscope it was postulated that a partial loss of hemoglobin had occurred from the individual cells in contradistinction to the approximately *all-or-none* loss which occurs from the cells as a result of suspension in hypotonic solutions containing electrolytes or small molecules [6, 9].

Since about 95 per cent of the anhydrous mass of the human red cell is hemoglobin, a lowering of dry mass may almost certainly be regarded as a close approximation of the hemoglobin loss. This paper reports preliminary experiments on the dry mass distribution of human red cells hemolysed in hypotonic electrolyte solutions containing dextran.

Methods.—Dyson [2, 8] and Johansson [4, 5] interference microscopes were used. Measurements of optical path difference and diameter were made visually using approximately monochromatic light (5461 Å) with the Dyson and white light with the Johansson instrument.

Cell suspensions.—Heparinized blood obtained from a vein was washed twice

in an isotonic salt solution, and then suspended in the following solutions, the final dilution of cells being 1:500.

(1*a*) 155 m*M*/l (130 m*N* NaCl, 5 m*N* KCl and 20 m*M*/l Na_2HPO_4–KH_2PO_4); 2×10^{-5} *M*/l Rose Bengal.
(1*b*) 40 m*M*/l (15 m*N* NaCl, 5 m*N* KCl and 20 m*M*/l Na_2HPO_4 KH_2PO_4); 7 g dextran/100 ml.
(2*a*) 77.5 m*M*/l (52.5 m*N* NaCl, 5 m*N* KCl and 20 m*M*/l Michaelis veronal acetate buffer).
(2*b*) 40 m*M*/l (15 m*N* NaCl, 5 m*N* KCl and 20 m*M*/l veronal acetate buffer); 5 g dextran/100 ml.

Immediately before measurements were made 1 part of suspension 2*b* was mixed with 1 part of 270 m*M*/l solution (245 m*N* NaCl, 5 m*N* KCl, 20 m*M*/l veronal acetate buffer) also containing 3.4×10^{-5} M/l CTAB (Cetyltrimethyl ammonium bromide).[1]

The pH of all solutions was 7.4. A narrow dextran fraction[2] (DR 172-IV; intrinsic viscosity $[\eta] = 0.239$, $M_n = 32.200$) was used. All salts were *pro analysi*.

The cells in 1*a*, 1*b* were measured with the Dyson and those in 2*a*, 2*b* with the Johansson instrument. Quartz slides and coverslips were used in the latter case but for the measurements made with the Dyson microscope desicoted glass slides and coverslips were used because quartz slides of sufficient area were not available. Cells in an isotonic solution containing no sphering agent, mounted on the Desicote surface did not all retain their biconcave shape, so it is possible that these surfaces were not quite without effect on the cells.

The cells were measured either as spheres or ellipsoids of revolution; in this way the total dry mass (M) in picograms (10^{-12} g) can be obtained from the optical path difference (Δ_M) of the central region of maximal thickness and the horizontal diameters (x, y) by the following equation [1]:

$$M = \frac{\pi}{6} x\, y\, \frac{1}{\chi} [\Delta_M + z(n_m - n_w)], \tag{1}$$

where χ is taken as 0.193 [10] for the whole red cell. When the suspending medium is such that all solvents can penetrate the cell and are present in equal concentrations within and without, the second term correcting for the difference between the refractive indices of the medium (n_m) and water (n_w) becomes zero and thus the thickness (z) is eliminated from the equation. In the case of cells in dextran this correction has to be applied.[3] In hypotonic salt and dextran the cells were in general round in horizontal section and from the appearance while they were settling on to the surface of the slide it was observed that the thickness seemed rarely less than about $\frac{3}{4}$ of the diameter. To obtain the dry mass of unhemolysed cells these were sphered photodynamically using Rose Bengal as a sensitizer; since such cells hemolyse later it was possible to measure them also as ghosts. A small series was measured with the Johansson instrument, and because the results obtained seem to fit well with the degree

[1] Kindly provided by Dr. A. V. Few.
[2] Kindly provided by Pharmacia Ltd., Uppsala.
[3] z was assumed to be equal to the smallest diameter.

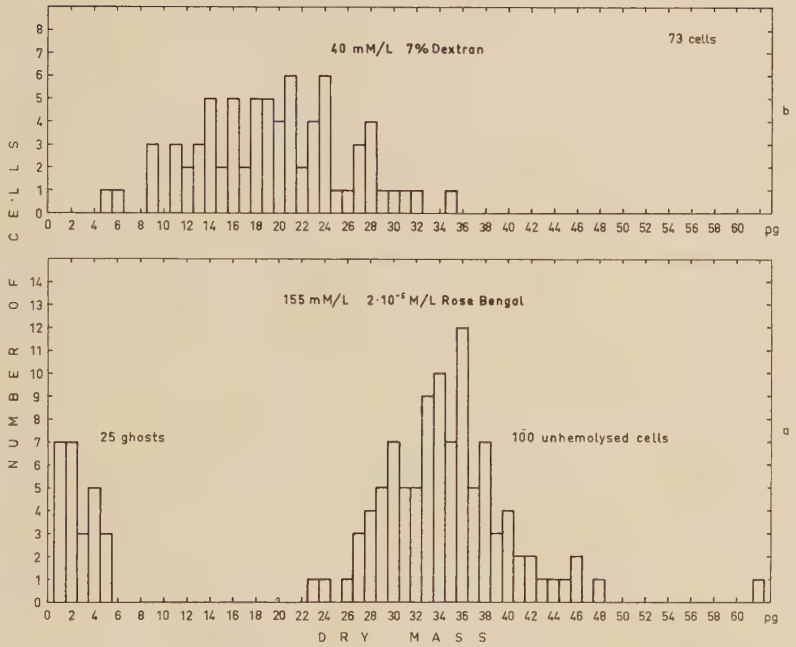

Fig. 1.—The dry mass distribution of human red cells measured with the Dyson interference microscope. (*a*) Unhemolysed cells in an isotonic medium and some of these cells after light hemolysis with Rose Bengal as a sensitizer; (*b*) in a hypotonic solution containing 7 g/100 ml Dextran with 51 per cent hemoglobin liberation. (This value and those mentioned in Fig. 2 were obtained from photometric estimation of the hemoglobin liberated in a red cell suspension. Per cent hemoglobin liberated (hemolysis) = 100 per cent—per cent inhibition. The inhibition values are expressed here as per cent hemolysis for comparison with the cells hemolysed hypotonically since the term inhibition refers only to the effect of dextran.)

of inhibition of hemolysis expected in red cells suspended in 40 mM/l salt and 5 per cent dextran solution [7] they are included here.

The material of Fig. 2 (*a*, *b*) is too limited to yield information as reliable as that of Fig. 1 (*a*, *b*) and we are accordingly making more extended observations. These experiments will take into account possible corrections for the condenser aperture in the Dyson microscope [3]. In order to reduce the error resulting from the necessity of assuming a value for the z diameter the dextran concentration was altered to 2.5 per cent and the tonicity to 155 mM/l for making the measurements. These dextran treated cells were compared with cells suspended in 77.5 mM/l solution which produced about 50 per cent hemolysis and thus a mixed erythrocyte ghost population. These cells appeared crenated in the isotonic solution and were sphered with CTAB which did not increase the amount of hemoglobin already liberated in the 5 per cent dextran 40 mM/l original solution.

Results.—The calculated dry mass distributions are shown in Figs. 1 and 2.

Discussion.—The distributions of the cells treated with 7 per cent dextran are quite different from those of the photodynamically sphered cells and their ghosts. Although the errors in the estimation of dry mass may be as great as 15 per cent,

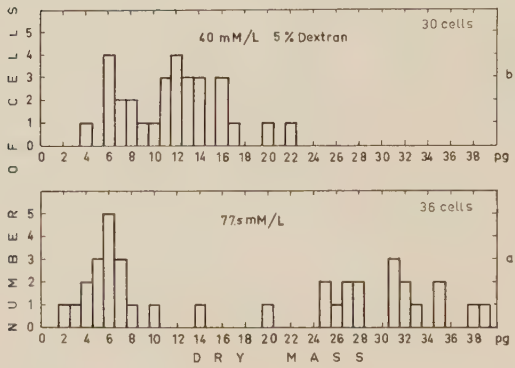

Fig. 2.—The dry mass distributions of human red cells measured with the Johansson interference microscope. (*a*) In a hypotonic solution producing about 50 per cent hemoglobin liberation; (*b*) in a hypotonic solution containing 5 g/100 ml Dextran with 66 per cent hemoglobin liberation.

mainly as a result of the error in estimating the diameter, these distributions are significantly different ($P < 0.001$). The photodynamically sphered cells have been assumed to be unhemolysed but the scatter in dry mass values is larger than might be expected. The optical path difference was, however, steady for several minutes while measurements were being made.

The inhibitory effect of dextran can be expressed in terms of either the residual dry mass or the residual hemoglobin. Cells suspended in a 40 m*M* l salt solution lose about 95 per cent of their hemoglobin [6].

If it is assumed that hemoglobin constitutes 95 per cent of the original dry matter of the erythrocyte and that the remaining 5 per cent which includes the stroma is not released, the degree of inhibition of hemolysis can be calculated as

$$\frac{\text{Mean dry mass} - 5 \text{ per cent of mean dry mass of unhemolysed cells}}{95 \text{ per cent of mean dry mass of unhemolysed cells}}$$

Table I shows the interferometric values for residual dry mass and calculated residual hemoglobin compared with the figures obtained by photometric estimation of the fraction of hemoglobin liberated from a suspension of cells in similar media [7].

Table I

Conc. of dextran (g/100 ml)	Microinterferometry		Photometry
	% residual dry mass	Calculated % residual hemoglobin	Residual hemoglobin
5	36	34.3	35
7	56	53.3	49

Both methods appear to give similar results as far as dextran concentrations of 5 and 7 per cent are concerned. The relation between the degree of inhibition and dextran concentration in suspensions containing 40 m*M*/l salt was found to be linear

up to at least 80 per cent inhibition [7] providing the dextran intrinsic viscosity exceeded 0.156 (M_N, 19.300). These results appear to support the hypothesis that in hypotonic electrolyte solutions containing dextran, hemolysis occurs by a partial liberation of hemoglobin from the single cell rather than in an approximately *all-or-none* manner.

We wish to thank Professor A. Engström for the kind loan of the Dyson microscope.

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PROTEINS OF ISOLATED MITOCHONDRIA FROM RAT LIVER

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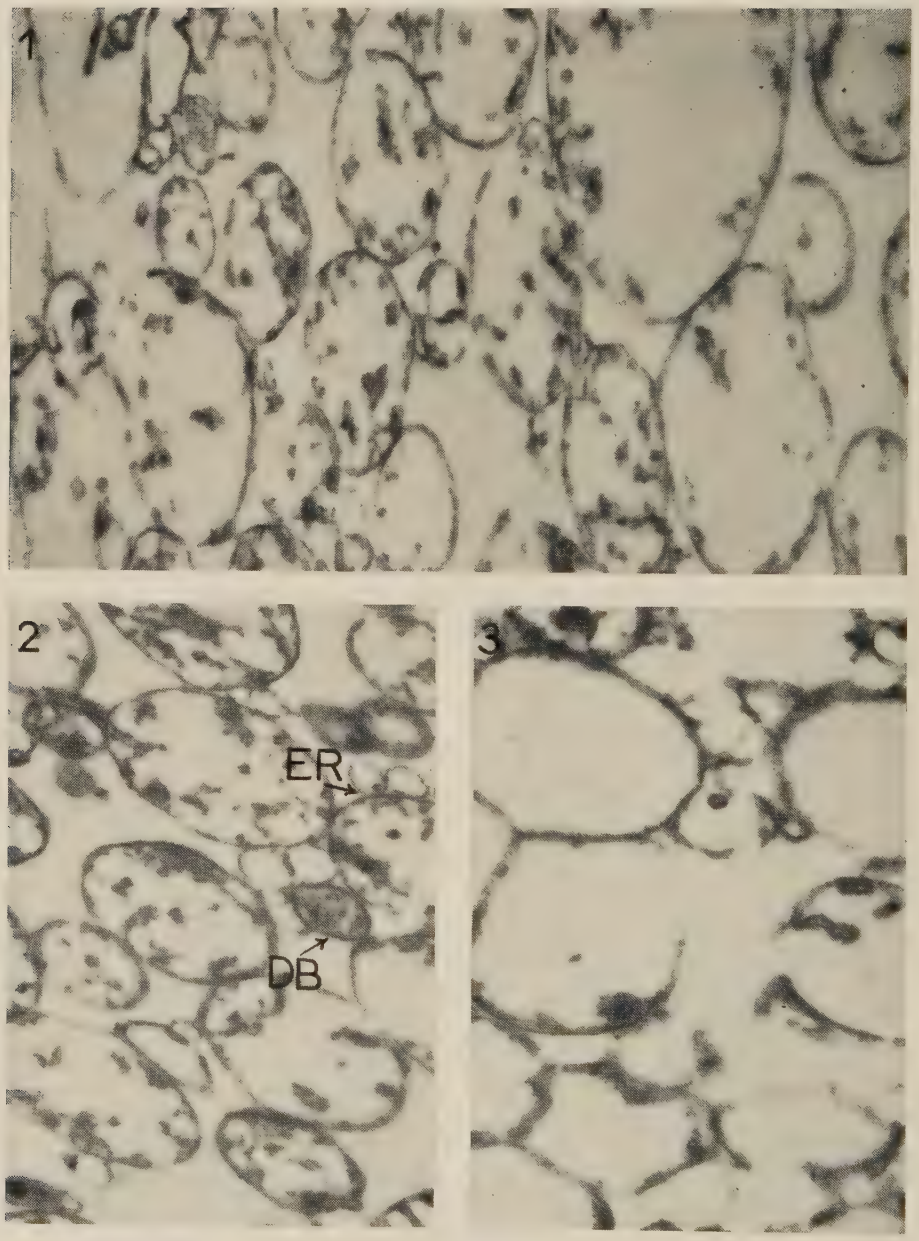
One of the main difficulties in the preparation of pure mitochondria is the removal of the fluffy layer: this fraction, rich in microsomes (containing a high proportion of ribonucleic acid and phospholipids), can, if not completely eliminated, bring about a notable change in the biochemical composition of the isolated mitochondria. We have a technique using liver-homogenate in 8.5 per cent sucrose which enables us completely to separate this fluffy layer [3]. It consists of suspending in solution the reddish fluffy layer which appears after centrifuging the first washing, while the yellow sediment of mitochondria remains closely applied to the side of the tube. This procedure involves a slight loss of mitochondria but this is unavoidable to obtain a really pure preparation.

Using an electron microscope we see that the purified preparations (Fig. 1) do not contain any ergastoplasmic structures, the small dense particles of which (100 to 150 Å) would prove the presence of microsomes [1, 5]. On the other hand, in a preparation in which the fluffy layer has not been removed (Fig. 2), these structures, as well as typical dense bodies, are seen [4, 6].

The concentration of total lipids and phospholipids in all purified preparations is found to be constant (Table I). This constant composition which has not been noticed previously would lead us to suppose the presence of a clearly definite cell



Figs. 1–3.—Ultra-thin sections of sediments, fixed in 1 per cent osmium tetraoxyde buffered at pH 7.4 and imbedded in butyl-methacrylate. ×23,000. (*1*) Preparation of pure mitochondria: there is neither ergastoplasmic formation (ER) nor typical dense body (DB). (*2*) Preparation of mitochondria without separation of the fluffy layer. Note the presence of ergastoplasmic formations (ER) and typical dark bodies (DB). (*3*) Fraction ($P_2 + P_3$). The mitochondrial membrane is generally disrupted. Photomicrographs kindly furnished us by Dr. C. Rouiller, Collège de France, Paris.

fraction. The figures in Table I show that, in the mitochondria, the phospholipids represent about 50 per cent of the total lipids.

The estimation of ribonucleic acid (RNA) in our preparations shows that the mitochondria contain RNA in slight but variable quantities. The analysis of our results enables us to claim that RNA really belongs to mitochondria and does not arise from microsomal contamination.

Fractionation of mitochondrial proteins

In view of liberating the mitochondrial enzyme-proteins, we have adopted conditions which tend to preserve the maximum enzymatic activity: we freeze the preparation in 34 per cent sucrose for one night at − 50°C; after allowing it to melt, we adjust the concentration to 8.5 per cent, with distilled water. The preparation is then homogenized and fractioned by differential centrifugation according to the principle given in Table I. It appears that this procedure leads to the rupture of the mitochondrial membrane (Fig. 3). But in spite of this damage, we notice that the fractionation is reproducible from one experiment to another.

(1) The fraction ($P_2 + P_3$) corresponds to 55 per cent of the protein nitrogen; it is richer in lipids than the intact mitochondria, but nevertheless the phospholipids are also equal to 50 per cent of total lipids. It contains practically all the mitochondrial RNA.

(2) The fraction P_4 existing in small quantities is composed of lipoproteins (the total lipids represent 50 per cent of the total weight). Moreover, it is very rich in succinodehydrogenase activity: with methylene blue, the activity per mg of nitrogen is three times as much as that of intact mitochondria. These results recall those of Green *et al.* [2], and Singer *et al.* [7] on the purification of the same enzyme from beef heart.

(3) The non sedimentable supernatant represents 30 to 40 per cent of the protein nitrogen. It is completely free of lipid and ribonucleic acid. The study of their enzymatic activities is in progress.

Table I

The figures in brackets represent the standard deviation. TL, Total lipids; PL, Phospholipids; RNA, Ribonucleic acid; N, Total nitrogen.

	mg TL	mg PL	Per cent of nitrogen	Per cent of RNA	Per cent of PL
Pure and intact mitochondria (20 m to 8500 g)	1.98 (0.04)	0.99 (0.06)	100	100	100
Fractions of mitochondria after treatment					
(1) P_2+P_3 (20 m to 8500 g)	2.92 (0.4)	1.45 (0.17)	55	80 to 94	80 to 87
(2) P_4 (30 m to 40,000 g)	6.36 (1.2)	3.27 (0.46)	3	2 to 4	4 to 6
(3) Supernatant		0.01	40	1	0 to 1

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STRUCTURES MYÉLINIQUES OBSERVÉES AU MICROSCOPE ÉLECTRONIQUE SUR DES COUPES DE GLOBULES ROUGES EN VOIE DE LYSE

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Les globules rouges vieillis ou altérés émettent des filaments (que l'on voit bien en fond noir ou en contraste de phase) qui restent souvent attachés à la surface globulaire. Ils donnent alors des images classiques « en tête de méduse » : Ces filaments ont été appelés *hématexodies* ou *formes myéliniques* [1, 11, 7]. On a montré que ces structures naissaient à partir des formations particulières observables au microscope électronique sur la surface des érythrocytes, appelées « figures en cratères » [2]. Figures en cratères et hématexodies semblent d'après leurs caractères morphologiques, comporter une très forte proportion de lipides. C'est ce qui les a fait ranger justement dans le groupe des « figures myéliniques ».

Dans des coupes de sang et de moelle osseuse, nous avons observé, non plus les figures myéliniques vues dans leur ensemble, mais des coupes ultra-minces de celles-ci.

Nous n'avons vu ces formations que dans des conditions particulières (lyse intracytoplasmique des globules rouges phagocytés et altérations de certains globules dans l'anémie de Cooley (cf. [3]). On voit sur des plages de 0,1 à 0,5 microns de diamètre, un ensemble de lames, coupées sous différentes incidences, au nombre d'une dizaine, dont la disposition contournée évoque l'image d'empreintes digitales (Fig. 1 et 2). L'épaisseur des lames varie de 60 à 80 Å, l'espace entre les lames varie de 75 à 120 Å. Ces aspects rappellent d'une manière frappante ceux que l'on voit dans des coupes ultra-minces de gaines nerveuses myéliniques (cf. [8, 5]).

L'origine de ces curieuses structures pourrait, à notre avis, être la suivante : dans un globule rouge normal il y a 90 % d'hémoglobine et 10 % de stroma. Celui-ci renferme environ 70 % de protéines et 30 % de lipides, essentiellement des phospholipides (lecithine, sphingomyéline et céphaline). Dans le globule altéré une partie du stroma se concentre en petites masses ovoïdes ou en croissant, placées généralement à la

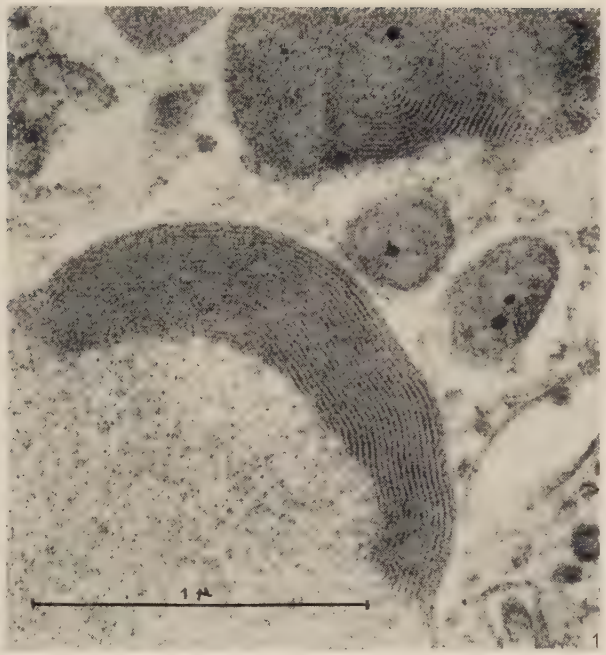

Fig. 1.—Structure lamellaire de deux fragments de globules rouges phagocytés par un macrophage de la moelle. (Grossissement 72.000.) En bas, à l'intérieur du globule, on remarque les fins granules ferrugineux (60 à 100 Å) [cf. 3].

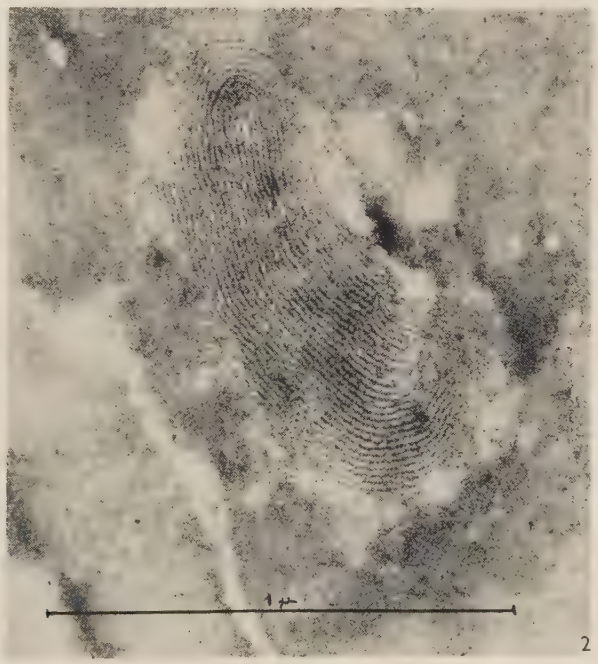

Fig. 2.—Structure myélinique d'un érythrocyte phagocyté par un macrophage de la moelle. (Grossissement 101.000.)

Tableau I

Epaisseur des lames (Å)	Espaces (Å)	N° des clichés
63	95	372 a
62	75	373 e
64	80	373 e
78	100	400 c
64	97	368 c
60	120	367 c
60	120	368 d

périphérie du globule. Celui-ci montre par ailleurs des signes certains d'hydratation (gonflement). Au niveau de ces résidus, riches en phospholipides, se déclanche le processus bien connu de la formation des « figures myéliniques ».

Les anciens travaux de Nageotte [10], ceux plus récents de Frey-Wyssling, de Dervichian et coll. [6, 4] etc., ont fixé les mécanismes physico-chimiques de ce processus. Un appel de molécules d'eau se produit au niveau des terminaisons OH des chaines lipidiques dipôles, qui se mettent en ordre en membranes bimoléculaires superposées et constituent les « lames formatives » (Nageotte) des figures myéliniques. L'eau s'accumulant entre ces membranes bimoléculaires lipidiques, les écarte les unes des autres.

Au microscope électronique, on voit, nettement dessinée en certains points, la coupe des lames formatives élémentaires, c'est à dire les membranes bimoléculaires des chaînes lipidiques. Ces lignes noires sont séparées par des espaces clairs correspondant aux parties hydrophyles non osmiophiles.

Les mensurations opérées sur des épreuves micro-électroniques paraissent confirmer cette explication. Les lamelles osmiophiles ont une épaisseur de 50 à 70 Å. Or, l'épaisseur théorique des doubles chaines lipidiques des corps du type lécithine serait de 63 Å, d'après Frey-Wyssling [6]. La concordance est donc bonne.

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CELLULOSE DEPOSITION IN ELONGATING EPIDERMAL CELLS OF *AVENA* COLEOPTILES[1]

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In recent publications, Wardrop [8, 9] has concluded that in elongating parenchyma and epidermal cells of *Avena* coleoptile, cellulose deposition is not confined to the cell tips, as Mühlethaler [4] has suggested, but takes place uniformly over the entire cell surface. This conclusion is based on autoradiographs of cells from 5-mm segments of coleoptiles which had been allowed to elongate for 12 hr. in a solution containing 10 μg of C^{14}-labelled glucose. The segments were macerated by alternate treatments with 0.1 *N* NaOH and 0.1 *N* HCl, thoroughly washed, dried on glass slides, and autoradiographs prepared by the stripping film method.

Ordin, Cleland and Bonner [5] have studied the incorporation of carbon-14 into cell-wall fractions of such *Avena* coleoptile segments treated for 3 hr. with C^{14}-labelled glucose. They found that after disintegration and thorough washing, the bulk of the carbon-14 incorporated into the cell walls was located in the fraction soluble in hot 0.05 *N* HCl (pectic substances) and to a lesser extent in the fraction soluble in cold 4 per cent NaOH (polyuronide hemicellulose) while about equal but still smaller amounts were incorporated into the fraction soluble in cold 17.5 per cent NaOH (non-cellulosic polysaccharides) and cellulose.

Wardrop does not give precise details of his maceration technique, but the alternate treatment with 0.1 *N* NaOH and 0.1 *N* HCl would probably have extracted most, if not all, of the pectic substances, and left behind for autoradiography not just the cellulose, but the large and significantly radioactive polyuronide-hemicellulose and non-cellulosic polysaccharide fractions as well. Since these are wall constituents, his conclusions would not be fundamentally altered by this correction. However, Wardrop makes no mention of how effectively maceration removed cytoplasmic proteins from the cells before they were autoradiographed. *Avena* coleoptiles are especially rich in protein [4] and significant amounts of carbon-14 would undoubtedly have been incorporated into this fraction as well as into the cell walls. Incomplete extraction of protein would make the autoradiographs very difficult to interpret.

When we macerated coleoptile segments by treatment for 24 hr. in 0.1 *N* NaOH followed by 24 hr. in 0.1 *N* HCl and examined by phase contrast microscopy, significant amounts of residual cytoplasm could still be seen as undissolved aggregations scattered throughout both parenchyma and epidermal cells. Even with 1.0 *N* reagents [2] the extraction of cytoplasm was poor. The conclusions that Wardrop draws from his autoradiographs concerning uniform deposition of cellulose and the absence of tip growth are therefore of somewhat doubtful significance. Castle [2], Wardrop

[1] N. R. C. publication number 4457.

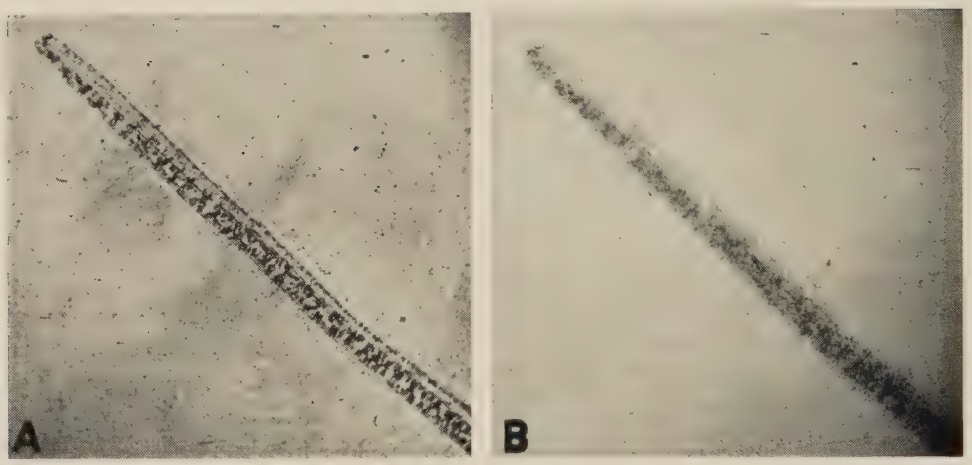

Fig. 1A.—Residual cellulose in the wall of an epidermal cell of *Avena* coleoptile treated with C^{14}-labelled glucose or C^{14}-labelled glucose-1-PO_4. Note thickened part of wall with transverse wrinkles along lower left-hand side. Phase contrast, ×240.
Fig. 1B.—Autoradiograph of same. Bright field, ×240.

[7], and Scott *et al.* [6], of course, have concluded on the basis of other evidence that elongation of parenchyma cells does not involve tip growth.

In an independent investigation of the same problem, initiated before Wardrop's papers were published, we prepared autoradiographs of epidermal cells from *Avena* coleoptiles which had elongated about 40 per cent during 21-hr. treatments in either C^{14}-labelled glucose or C^{14}-labelled glucose-1-PO_4. Although we subjected these cells to a much more rigorous extraction than did Wardrop, to insure that little but α-cellulose could have remained, our autoradiographs of cells from both of these treatments showed the same uniform longitudinal distribution of carbon-14 throughout the wall that he has reported (Fig. 1). Fig. 1 also clearly shows the presence of more carbon-14 in the thickened outer wall, confirming the impression given by Wardrop's Plate 2, Fig. 5 [9].

In our experiments, 18 coleoptiles, about one centimeter long, were excised and allowed to stand for 21 hr. in darkness, at 25°C, base down in a vial containing 0.2 ml of a 1 per cent solution of uniformly C^{14}-labelled glucose (3.2 μc). Another lot of excised coleoptiles was similarly treated with 0.2 ml of a 2 per cent solution of uniformly C^{14}-labelled potassium glucose-1-phosphate (4 μc). After thorough rinsing, strips of epidermis were peeled from the excised coleoptiles and extracted as follows: (1) 1:1 95 per cent ethanol : concentrated HCl, 5°C; (2) 0.5 per cent ammonium oxalate, 5°C; (3) 17.5 per cent KOH, 25°C. The duration of each step was 24 hr. including thorough rinses between. In a control experiment in which coleoptiles and primary leaves that had been disintegrated in a Waring blendor and extracted with 80 per cent ethanol were treated by the foregoing procedure, the nitrogen content was reduced from 3.6 to 0.16 per cent on a dry weight basis. The cells of the extracted epidermal peels had very delicate walls and cytoplasm was no longer microscopically detectable. A suspension consisting mostly of single cells was prepared by agitating the extracted

peels in a Mickle [3] sonic disintegrator without ballotini for 1 minute. Drops of cell suspension were smeared over Kodak NTB Autoradiographic plates, dried *in vacuo*, stored for 28 days at 13.5°C, and developed at 68°F.

The authors are indebted to Professor G. Krotkov, Dept. of Biology, Queen's University, Kingston, Ontario, for the generous gift of the C^{14}-labelled sugars.

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GOLGI STRUCTURE OF ADENOPITUITARY CELLS REVEALED BY REFRACTOMETRY AND MICROINCINERATION

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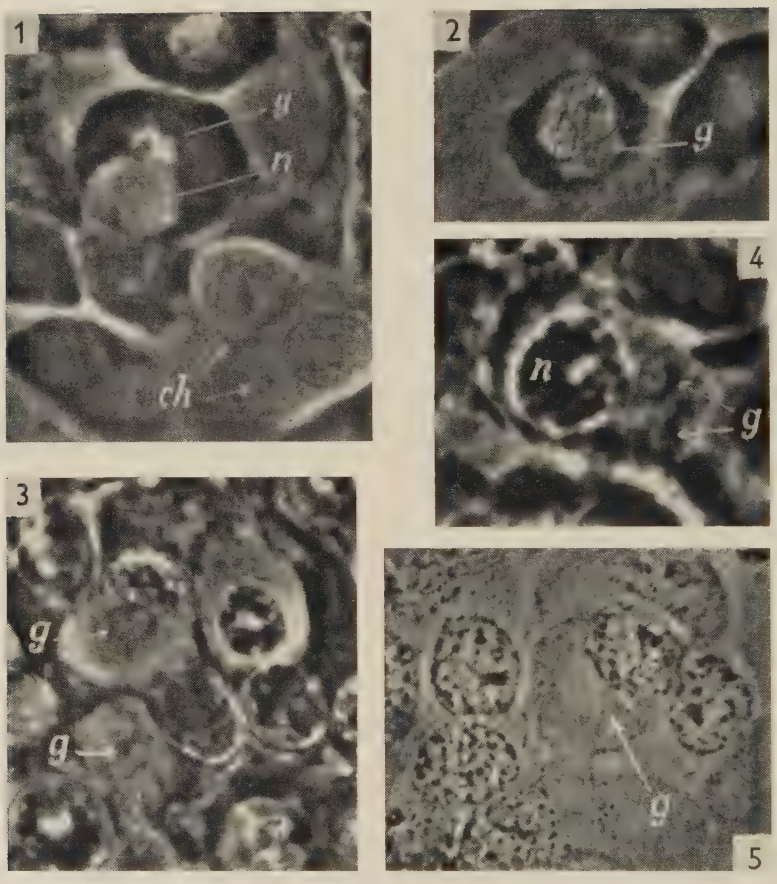
It has been found that the different types of cells in the adenopituitary can be differentiated by refractometric technique, and their protoplasmic components such as granules, vacuoles and mitochondria may also be studied.

Particular consideration has been given to the Golgi structure—its refractive index (RI) and mineral constitution. Investigations have been made in the correlation of its refractive index and mineral residues.

Material and Methods

Normal human pituitary provided the main material, supplemented by cat, guinea pig, rat and rabbit tissues. The samples were prepared both by routine fixation and by freeze-substitution [4]. The refractometric technique was as before [3, 5, 9]; it entailed the examination and photography of unstained sections by phase microscope. These were then incinerated. Thus both the phase appearance and the spodogram of the same section of a given cell could be compared.

Thin (2–3 μ) sections were mounted on coverslips, deparaffinized in xylol, and mounted on slides in media of known RI. The cells could easily be differentiated into α, β and chromophobes. Chosen cells were marked and photographed in the different media, after which the coverslip bearing the section was removed and washed in



Photomicrographs Nos. 1–4 were of unstained sections in phase illumination, × 95 oil immersion objective. α,α-cells; β,β-cells; *ch*, chromophobes; *b*, background; *g*, Golgi structure; *n*, nucleus.

Fig. 1.—Optical differentiation of adenopituitary cells mounted in xylol (RI 1.497). α-cell in the centre showing Golgi structure as a negative, on top of nucleus. Chromophobes (bottom right) showing mitochondria. Cat. Freeze-substitution.

Fig. 2.—α-cell mounted in xylol (RI 1.497). Note in the centre of the Golgi area phase positive centrosome. Cat. Freeze-substitution.

Fig. 3.—α-cells and chromophiles mounted in Benzyl-benzoate (RI 1.5692). Two α-cells showing phase positive Golgi rings. Their protoplasm is phase negative in comparison to the background. Rabbit, pars distalis, fixed in Susa fluid.

Fig. 4.—α-cell mounted in Benzyl-benzoate (RI 1.5692) showing phase-positive rings of the Golgi structure. Rabbit, pars distalis, fixed in Susa fluid.

Fig. 5.—Incinerated section showing the appearance of the ash of cat adenopituitary. Freeze-substitution. Phase illumination, × 95 oil immersion objective.

xylol. It was then transferred to the microincineration apparatus; finally the spodogram of the marked cells was examined and photographed under oil immersion objective either with the phase microscope [8], or in incident light (Ultropak of Leitz).

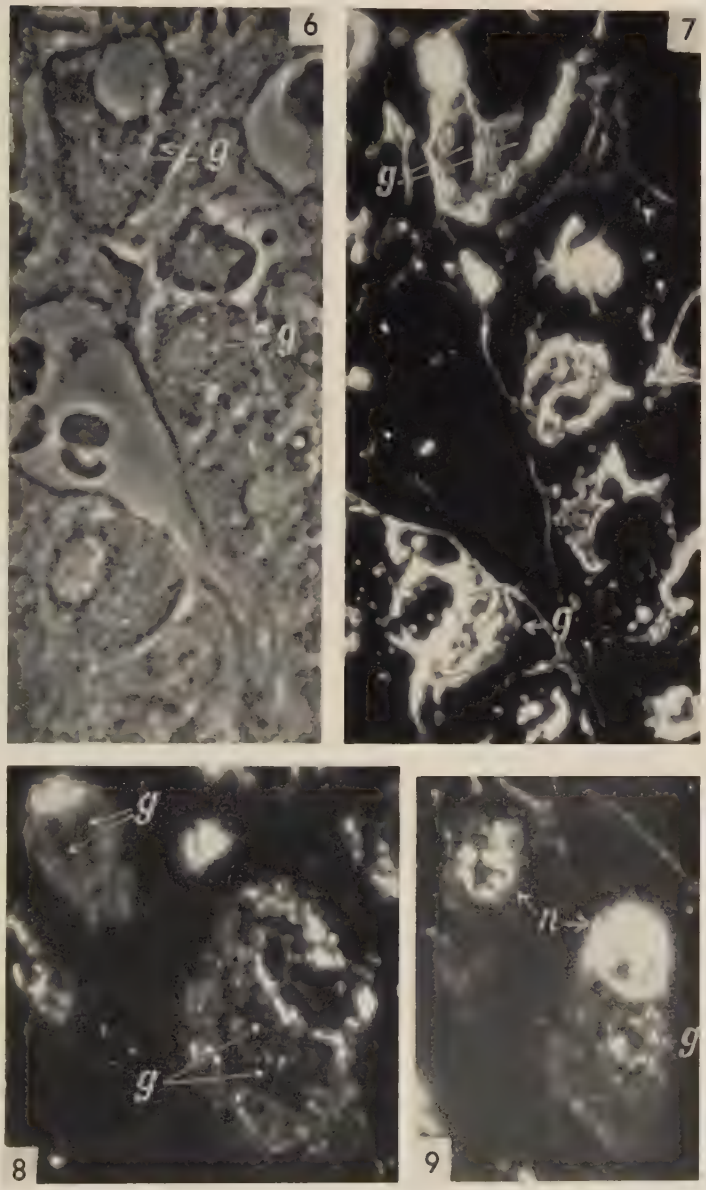


Fig. 6.—Human pituitary α-cells showing Golgi structure. Section was mounted in chlorobenzene (RI 1.5243). 10 per cent formalin fixation.
Fig. 7.—The same section as above after incineration. Canalicular network is visible: it corresponds to the Golgi structure. Incident light (Ultropak of Leitz) ×100 oil immersion objective.
Fig. 8.—Incinerated section showing the ash of human chromophiles and their ash-free Golgi area. 10 per cent formalin fixation. Incident light (Ultropak of Leitz) ×100 oil immersion objective.
Fig. 9.—Incinerated section showing the ash of human β-cells. 10 per cent formalin fixation. Incident light (Ultropak of Leitz) ×100 oil immersion objective.

Results

Phase microscopy and RI.—When examined in phase-contrast illumination, the Golgi structure in unstained adenopituitary cells was seen as a group of small round spaces, canaliculi or rings, either adjacent to the nucleus or dispersed in the protoplasm (Figs. 1 4, 6). This corresponds to what has been seen after impregnation or staining as described by Severinghaus [14], Atwell [2], Romeis [12], Wolfe [15], Arzac and Flores [2], Rinehart *et al.* [11], and others. The Golgi structure seemed to consist of free spaces in the protoplasm and its RI corresponded with that of background. It was low and phase-negative in media of RI 1.50–1.55; high and phase-positive in media above RI 1.56. The amorphous protoplasm, mitochondria, granules, nucleus and nucleoli were in reversed contrast. The image of the Golgi structure was at times exaggerated by "optical membranes" [10].

Microincineration.—Scott [13] quoted by Hintzsche [6, 7] described three types of cells in a spodogram. Nothing, however, has been mentioned about the mineral ash of their Golgi structures, nor apparently is there any reference to this in regard to any other animal cell.

The mineral deposits encountered in the present material corresponded not only to protoplasm, nucleus, nucleolus and chromatin, but also, with chromophiles, to the Golgi structure. These cells, usually rich in granular ash, showed an ash-free zone as a ring or round space near the nucleus. In some cases, this area had a little ash (Fig. 5) but much less than in the surrounding cytoplasm. In other cells where the ash is less, as in β-cells, the protoplasm near the Golgi canaliculi contains more ash than in the periphery (Fig. 9). The Golgi rings and canaliculi in thicker sections (3–6 μ) were more pronounced after incineration owing to the shrinkage of the surrounding cytoplasm (Figs. 7, 8).

Most of the incinerated chromophobes left but a little ash corresponding to their nucleus, and only traces to their protoplasm.

Summary

Fixed but unstained sections of pituitary mounted in media of different RI have been examined in phase illumination. Optically differentiated chromophiles showed canaliculi and rings corresponding to the Golgi structure. As the RI of the Golgi structure was the same as that of the background, canaliculi seem to have represented empty spaces. These canaliculi were seen in chromophiles even after incineration; they were ash-free, while remaining protoplasm contained abundant mineral deposits.

A new method was described for the identification of cells and cell components in a single section before and after incineration.

I wish to express my sincere thanks to Dr. N. M. Hancox for his kind help in preparing the manuscript and for the loan of an Ultropak; to Dr. B. Boczarow (Birmingham) for kindly providing human pituitary samples; and to Mr. R. McV. Weston for the loan of an immersion oil objective to the Ultropak.

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REGENERATING PERIPHERAL NERVE SHEATHS FOLLOWING WALLERIAN DEGENERATION[1]

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As part of a general investigation of the ultrastructure of myelin, a study of Wallerian degeneration was undertaken utilizing the sciatic nerves of the mouse. The nerves were divided with sharp scissors and allowed to retract. There was no bleeding as the nerve was cut. In a few animals the sectioned nerve ends had rejoined in a fusiform swelling in 3 to 4 weeks after operation. These animals still displayed a degree of paralysis which was similar to that noted shortly after section of the sciatic nerves. It may be assumed, therefore, that regeneration was still incomplete at the time of this study.

The zones of fusiform swelling were studied with the light microscope, utilizing preparations stained with Sudan III for fat and by the Spielmeyer technique for myelin. Material for the electron microscope (RCA-EMU-3A) was fixed in veronal buffered osmic acid (Palade's solution) and sectioned on the Servall Porter-Blum ultra-microtome.

The stained sections revealed only a few droplets of fat either free or in phagocytes. The myelin sheaths were pale, thin, and somewhat irregular in width and in staining quality. The whole area of swelling was hypercellular, due principally to large numbers of Schwann cells and fibroblasts.

In the electron micrographs, numerous young myelin patterns were prominent. The appearance of regenerating myelin was essentially similar to that of embryonic

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[3] With the technical assistance of M. Besen.

nerve as described by Geren [4]. Fig. 1 illustrates an early stage of myelin regeneration with the prolonged mesaxon [2] wrapped loosely around two-thirds of the axon's circumference. At its outer end the mesaxon was continuous with the Schwann cell plasma membrane, while medially it was continuous with the outer layer of the axon's double shell of which the inner membrane is the axolemma. The configuration of this neurite was similar to that of a C fiber, but the latter are usually more superficially placed in the Schwann cell and are accompanied by other fibers [5]. It is therefore more probable that this was an axon destined for myelination. Since the medullated axons occupied relatively little of the hypertrophic Schwann cell cytoplasm, the mesaxons were elongated and clearly delineated even in the later stages of myelination seen in Figs. 1 and 2.

Geren has suggested that the spiral structure of myelin might be due to rotation either of the axon or of the Schwann cell surface. The first seems awkward considering the length of an axon. Hess has objected to the latter explanation because he found occasional adult Schwann cells containing two medullated axons with sheaths of different thicknesses. Both objections could be met by postulating a locus of growth along the mesaxon. For example, if lipoprotein elements were inserted primarily at the innermost aspect of the mesaxon, only that layer immediately surrounding the axolemma need rotate and the myelin would be drawn around into the spiral pattern (Fig. 3).

Occasional bizarre myelin patterns were met as in Fig. 4 in which are seen several concentric myelin spirals each running into the same membrane immediately exterior to the axolemma. Although the multiple mesaxons are not apparent, all of the spirals have evidently arisen in the single Schwann cell. This configuration does not seem to fit the usual concept of embryogenesis. It is possible, however, that this formation may represent a stage in the genesis of a node of Ranvier as seen in tangential section [6].

Naked axons, without surrounding Schwann cell cytoplasm, were not seen in our material [1, 7]. This was not considered definitive proof of their absence in regeneration since the earliest stage of regrowth was not examined in these experiments. Schwann cells containing several, small non-medullated axons (Fig. 5) were more common than in normal nerves.

The Schwann cell cytoplasm was abundant at the stage of regeneration studied here. The particulate component of ergastoplasm was prominent while the membranous portion was often lacking. The mitochondria were similar to those present in the axoplasm, although they were usually somewhat less dense. The cytoplasm was bounded by a double membrane; the inner, more dense layer is the plasma membrane which, invaginated by the axon, formed the mesaxon. The outer or basement membrane [3] was less dense and, where it was cut tangentially (Fig. 2), was seen to be

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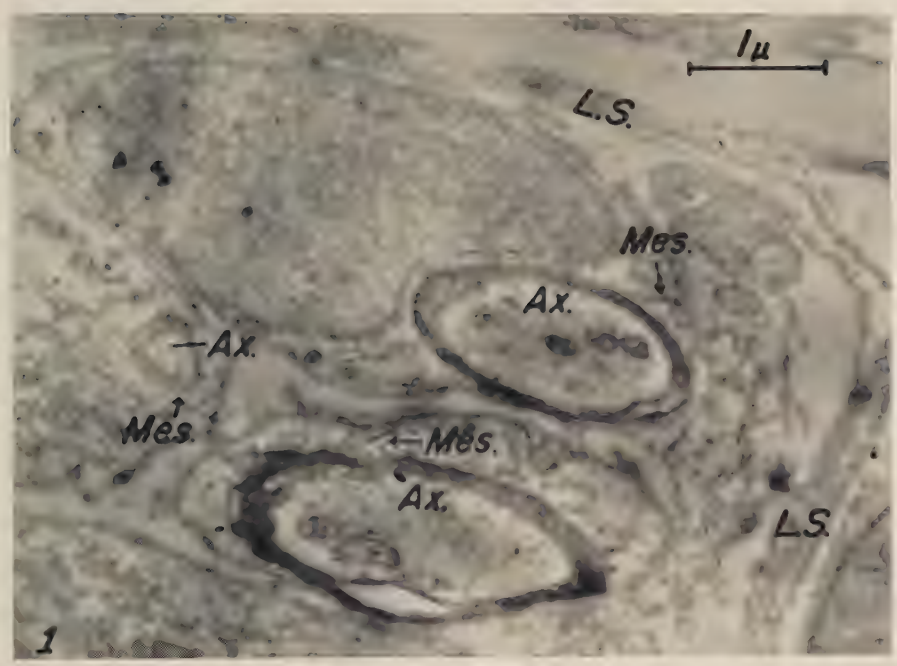
Abbreviations.—*Ax*, axon; *BM*, basement membrane; *Col*, Collagen; *LS*, laminar sheath or perineurium; *Mes*, mesaxon; *PlM*, plasma membrane; *ScCyt*, Schwann cell cytoplasm; *ScNuc*, Schwann cell nucleus.

Fig. 1.—A very young axon is seen at left. The two myelin sheaths have prominent mesaxons. The laminar sheath is at the top and right.

Fig. 2.—A three-layered myelin spiral is present at left between several unmyelinated axons. The basement membrane is seen to be continuous with the adjacent collagen at the top.


1μ
L.S.
Mes.
Ax.
Ax.
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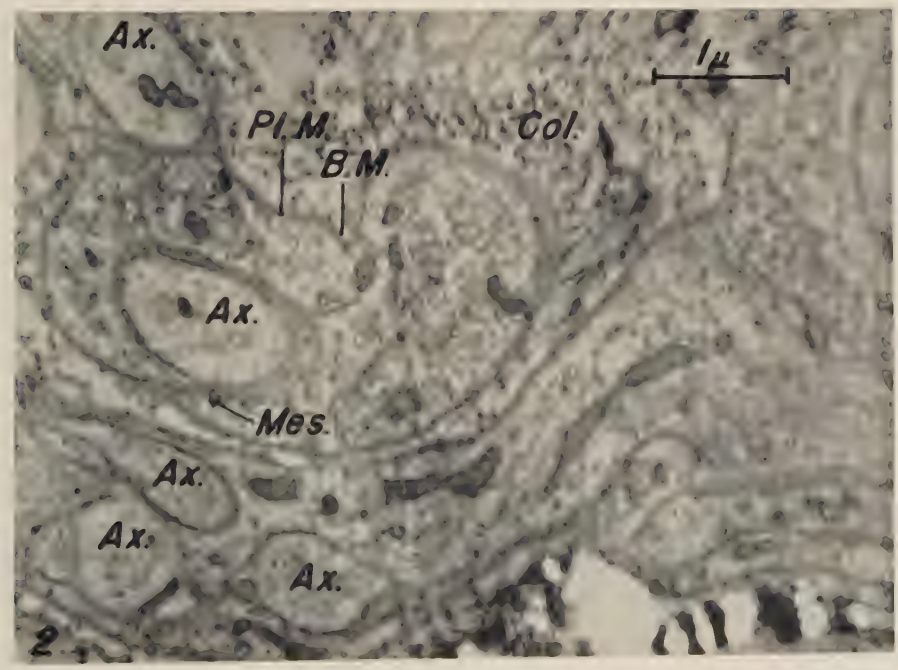
Mes.
Ax.
L.S.
1


Ax.
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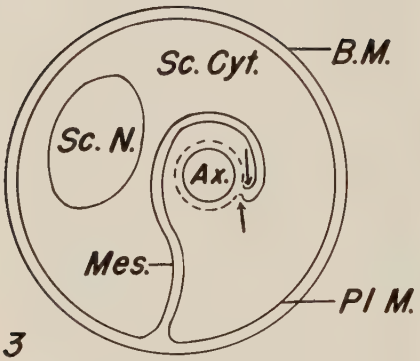
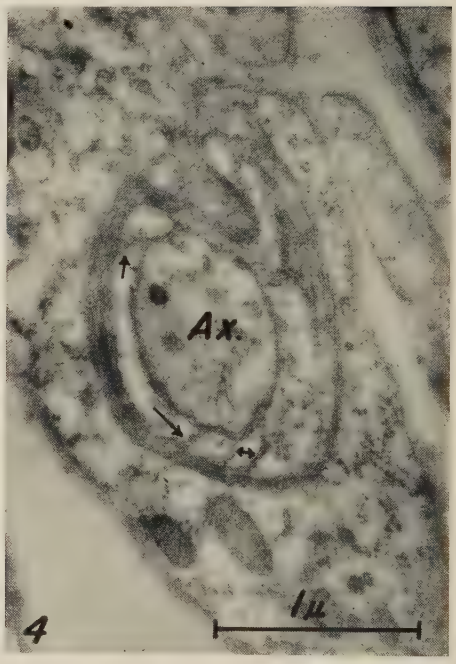
Pl.M.
B.M.
Col.
Ax.
Mes.
Ax.
Ax.
Ax.
2


Sc. Cyt.
B.M.
Sc. N.
Ax.
Mes.
Pl M.

3


Ax.
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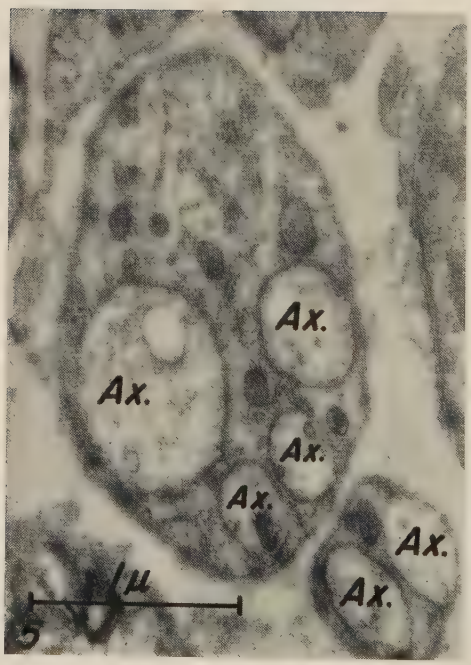
4


Ax.
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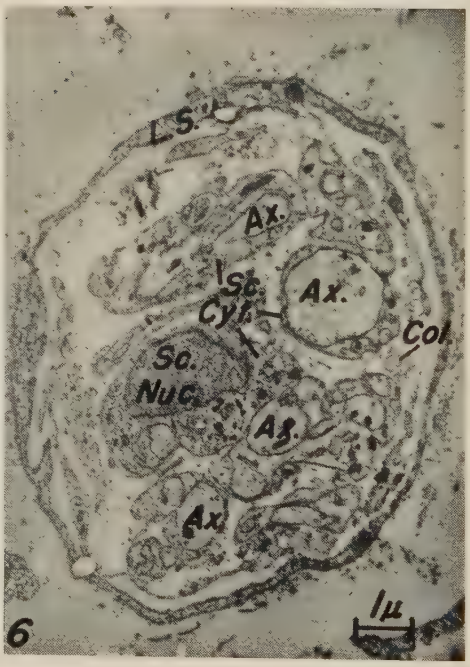
5


L.S.
Ax.
Sc. Cyt.
Ax.
Col.
Sc. Nuc.
Ax.
Ax.
1μ

6

continuous with collagen fibers. The sheath of Key and Retzius corresponds to those collagen fibrils which are most closely applied to the basement membrane. The latter was continuous over the defect in the plasma membrane where the mesaxon took origin. This was constant whether C fibers or medullated axons were involved. The basement membrane, therefore, must have been laid down after the stage in which the axis cylinder indents the surface of the Schwann cell. The neurilemma may thus be defined as consisting of three elements: first, the Schwann cell and its plasma membrane; second, the extracellular membrane; third, the immediately adjacent collagen fibers. The endoneurium is made up of the remaining collagen fibers and connective tissue cells.

The regenerative perineurium or laminar sheath was found exterior to the previously described elements. As seen in Figs. 1 and 6, it consisted of a thin band of cytoplasm in which were several continuous double membranes. This tissue is apparently made up of narrow cytoplasmic extensions of overlapping fibroblasts.

Summary.—Regenerating peripheral nerve sheaths have a fine structure very similar to that seen in normal embryonic nerve.

The authors are indebted to Dr. Harry M. Zimmerman, Chief of the Laboratory Division of Montefiore Hospital, for his advice and support.

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Fig. 3.—Diagram of embryonic or regenerating myelin (after Geren). The arrows indicate the proposed locus of growth. Only the broken line need rotate as the mesaxon elongates and the spiral thickens.

Fig. 4.—The myelin lamellae (arrows) each continue into the innermost layer of the sheath.

Fig. 5.—Schwann cell containing 4 unmyelinated axons. The mesaxons are very short and broad.

Fig. 6.—Low power view of a regenerating bundle containing several Schwann cells and surrounded by the laminar sheath.

IDENTIFICATION OF MUCOPOLYSACCHARIDES BY MEANS OF FLUORESCENT BASIC DYES

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Many attempts have been made with regard to the identification and differentiation of mucopolysaccharides in histological sections [5]. Yet it can hardly be said that the methods published so far are very satisfactory: lack of specificity is one of their principal drawbacks [5]. One of the most widely used methods depends on the metachromasy of sulfated mucopolysaccharides with basic dyes such as toluidine blue or thionin [6, 8, 4]. It seems that in this respect metachromasy-fluorescent dyes offer many advantages. In the monograph of Haitinger [3] the multicoloured pictures effected with only one dye are very vividly described. Some years ago we were much impressed by the many colours discernible in histological sections treated with dyes of acridine type, especially by the outstanding bright colour of the cartilage. Recently we again took up the study of interactions between fluorescent basic dyes and acidic compounds in sections. This preliminary note will give some results of these studies

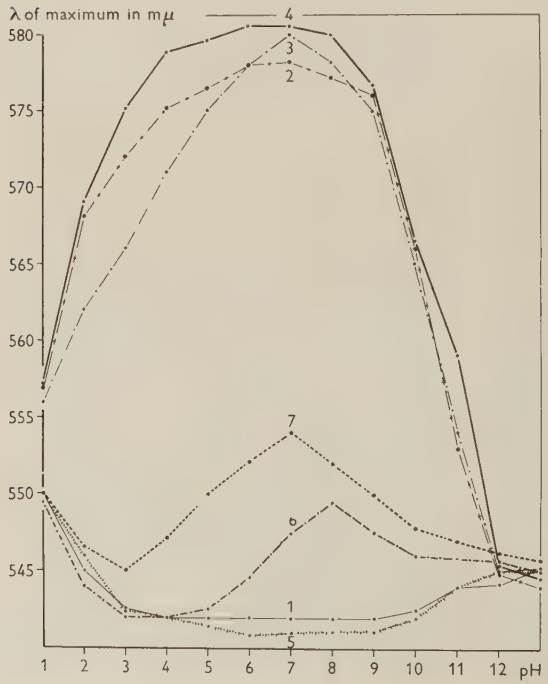

Fig. 1.—Fluorescence spectra of coriphosphin alone (*1*) and combined with hyaluronic acid (*1*), glycogen (*1*), DNA (*1*), mucoitin sulfuric acid (*2*), chondroitin sulfuric acid (*3*) and heparin (*4*).

Ch. M. A. Kuyper, "Identification of mucopolysaccharides by means of fluorescent basic dyes", this journal **13**, 198:
Pp. 198–9: *The charts referring to the captions for Figs. 1 and 2 to change place.*

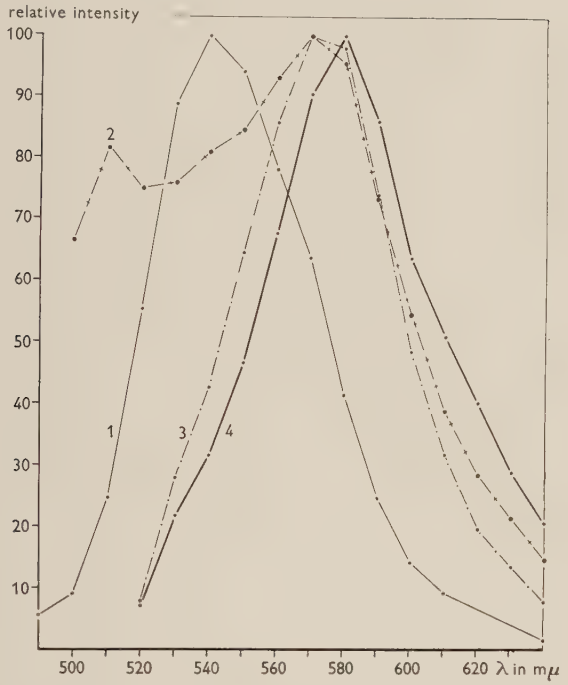

Fig. 2.—Variation with pH of wavelength of maximum fluorescence of coriphosphin alone (*1*) and in combination with mucoitin sulfuric acid (*2*), chondroitin sulfuric acid (*3*), heparin (*4*), hyaluronic acid (*5*), DNA (*6*) and RNA (*7*).

pertaining to coriphosphin and its coloration of mucopolysaccharides and nucleic acids.

In this study the fluorescence spectra were analysed from combinations *in vitro* of coriphosphin (Grübler) with mucopolysaccharides and other compounds in solution. The exciting light was produced by a Philips 57201E Hg-lamp, from which the near ultraviolet radiation was isolated by means of filters. The fluorescent light was collected on a slit 1 mm wide, passed through a Balzers variable interference wedge filter and the resulting narrow spectral bands were measured with a RCA 931A photomultiplier tube. A yellow filter was interposed to absorb any stray ultraviolet radiation from the tubes. Hyaluronic acid was prepared from human umbilical cords [1], chondroitin sulfuric acid from bovine trachea [2], heparin and mucoitin sulfuric acid were from N. V. Organon, Oss, Netherlands,[1] DNA, RNA and glycogen commercial preparations (Fishes Scientific Co.). All substrates were dissolved in water (1 mg/ml) and mixed with 5 ml coriphosphin R, in water (0.1 mg/ml). When working at different pH glycocoll buffers [7] were used.

When constructing the graphs the maximum intensity of each spectrum was set at 100. In Fig. 1 the fluorescence spectra of several combinations are shown. Glycogen, DNA and hyaluronic acid do not change the spectrum of coriphosphin; RNA and mucoitin sulfuric acid shift the colour from green to yellow, chondroitin sulfuric acid to orange and heparin to red. The shifting of the maximum alone is not an

[1] We wish to express our gratitude to this firm for the gift of these substances.

adequate measure for the change in colour: the fraction of red light e.g. is of much greater importance for the subjective colour impression. Also it should be borne in mind that the spectral sensitivity of the RCA 931A photomultiplier tube, especially in the red portion of the spectrum is much lower than that of the human eye. Visually, the several colours are clearly different. The fluorescence colours from the main mucopolysaccharides are thus clearly different, but at the pH used (7.0) some overlapping with other acidic components does occur. Therefore the fluorescence spectra of the substrate-coriphosphin mixtures were measured at different pH's. In Fig. 2 the results are shown and it is clear that at pH 4 a sharp differentiation between mucoitin sulfuric acid, chondroitin sulfuric acid and heparin can be effected without interference from DNA or RNA. Also other fluorescent basic dyes: acridine-orange (Grübler, Gurr) and euchrysin (Grübler, Gurr) give different colours with mucopolysaccharides. The differences in colour, however, between the several substrates are not so clear as with coriphosphin; nor do changes in pH effect the colours to the same extent.

Further work is now in progress to test the possibilities of coriphosphin as a specific histochemical tool, as well as on the mechanism of the polychromasy. The first results show clearly that also in sections a good identification of sulfated mucopolysaccharides can be obtained.

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ULTRASTRUCTURE OF THE NUCLEAR MEMBRANE OF A GREGARINE PARASITIC IN GRASSHOPPERS[1]

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Evidence from studies on the ultramicroscopic structure of the nuclear membrane is rapidly accumulating to show that in some cells it is composed in part of pores,

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nodes or annular structures. This condition of the nuclear membrane has been observed in certain tissues of mammals [10, 20, 24, 27], birds [17], amphibians [9, 11, 12, 18, 19, 22], insects [3, 4, 6, 13], annelids [8], molluscs [23, 26], echinoderms [1] and protozoa [5, 14, 15, 21, 25]. Not all authors are agreed that the annuli are porous structures [17]. Some have observed the nuclear envelope to be composed of two closely associated membranes [16], the outer one of which is sometimes continuous with membranes of the endoplasmic reticulum [27]. Electron micrographs of material apparently fixed in the process of traversing the pores have been published [3].

In the course of an electron microscope study [7] on the surface structure of the protozoan *Gregarina melanopli* (sometimes referred to as *Gregarina rigida* [2]), which is parasitic in the gut of the grasshopper *Melanoplus differentialis*, it was observed that the nuclear envelope possessed relatively large and distinct pores or annuli. These observations seem of sufficient significance to report, since they not only lend further evidence to the view that the nuclear membrane often shows a porous structure, but also that in some protozoa the nuclear envelope is intimately associated with a porous cortical layer of the nucleus.

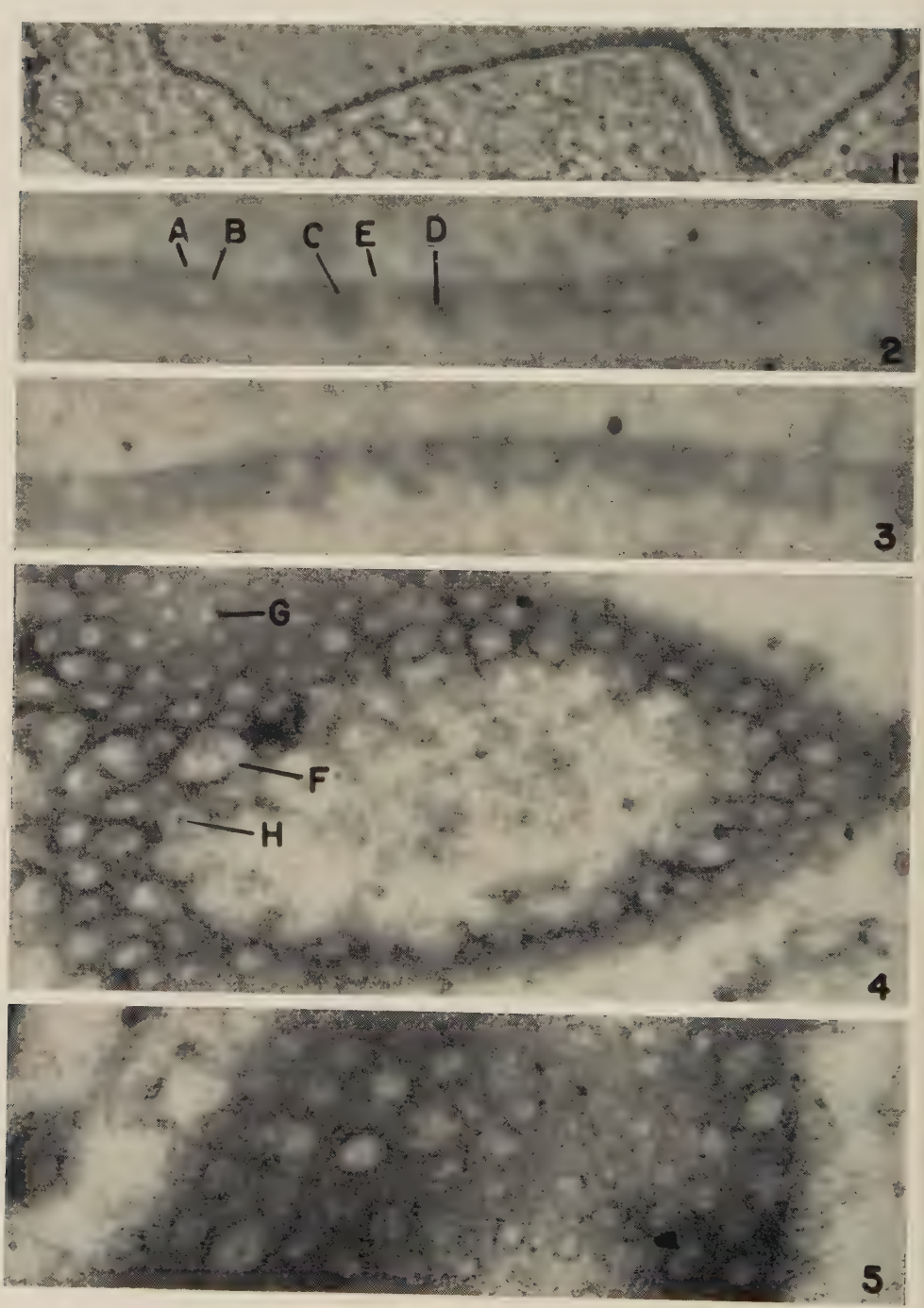
Material and methods.—The intestine of the grasshopper was opened and the sporants transferred directly to one per cent osmium tetroxide buffered at a pH of 7.3. They were fixed for 30 to 60 minutes, washed quickly in water, dehydrated rapidly and embedded in methacrylate. Thin sections were cut with a Porter-Blum microtome and studied by aid of an RCA model EMU 2B electron microscope.

Observations.—For a detailed description of *Gregarina melanopli* the reader is referred to Allegre [2]. It will suffice to point out that the animal is composed of an anterior non-nucleated portion, the protomerite, and a posterior nucleated portion, the deutomerite. Often the sporants associate in pairs with the deutomerite of the primite being attached to the protomerite of the satellite.

A relatively low power electron micrograph of a slightly tangential section of the nuclear envelope is shown in Fig. 1. The envelope at this magnification appears to be comparatively thick and to possess a nonhomogeneous structure. Under higher magnification it is revealed to possess the usual two porous membranes seen in a considerable number of nuclei and, in addition, a third relatively thick (900 Å) cortical layer is also present. The relationship of the components of the nuclear envelope to one another is best seen in sections cut perpendicular to the nuclear surface (Figs. 1-3). The labels in Fig. 2 also apply to Fig. 3. In these figures may be observed layers arranged from the outside inward as follows: (1) an outer relatively dense porous membrane measuring about 80 Å in thickness (*A*); (2) a less dense intermembrane space about 120 Å in thickness (*B*); (3) an inner porous membrane (*C*) comparable to the outer membrane (*A*) in structure and thickness and (4) a cortical honeycomb relatively thick (900 Å) and porous layer (*D*).

In contact and intimately associated with the inner membrane *C* is the cortical differentiated nuclear layer *D* which is relatively thick, porous, and possessed of a fine fibrous structure. It appears to represent a natural and consistent cortical differentiation of the nucleus.

Perhaps the most striking characteristic of the nuclear envelope and associated underlying cortical layer is the presence within them of relatively well-defined pores. These extend through both the nuclear envelope and the underlying cortical layer (Figs. 2 and 3). In sections cut perpendicular to the nuclear surface the pores appear


A
B
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D
G
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cone-shaped with the apex extending through the nuclear envelope, coming in contact with the cytoplasm and the base extending through the underlying cortical layer, where it is in contact with the nuclear substance (Figs. 2 and 3). The width of the pore at its apex is approximately 500 Å and it increases until near the base it approximates 1500 Å or more.

Sections tangential to the nuclear surface and through the cortical layer reveal quite clearly the honeycomb pattern of this layer (Figs. 4 and 5). The difference in width of the pores varies with the level of section across them, i.e., those cut near the inner surface of the cortical layer are widest (*F*) and those cut near the outer surface of the nuclear envelope the narrowest (*G*).

Of interest in these figures, also, is the presence near the center of certain pores of a granular appearing body (*H*). It may represent some form of closure or diaphragm of the pores or possibly only coagulated materials caught in transit through them (see also [13]). The ground substance of the cortical layer appears in some preparations to possess small granules (Fig. 5).

Discussion and conclusions.—The nuclear envelope of *Gregarina melanopli* seems to possess a structure comparable to that of *Amoeba proteus* as described by Pappas [21] (see also [14]). That is, it contains an outer porous nuclear envelope composed of two membranes separated by an intermembrane space characteristic of many animal nuclei. In addition, associated with it is a porous cortical nuclear differentiated layer. It is not known how extensively this cortical nuclear layer occurs in animals; however, as demonstrated here, it is not confined to *Amoeba proteus*.

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Fig. 1.—Low power of slightly tangential section through nuclear envelope. Even at this magnification the pores are evident. The nuclear substance is above the membrane and the cytoplasm below. ×8,000.

Figs. 2–3.—Sections cut perpendicular to the nuclear surface. Labels in Fig. 2 also apply to Fig. 3. The outer membrane of the nuclear envelope is shown at *A*, the intermembrane space at *B*, the inner membrane of the nuclear envelope at *C*, the nuclear pore at *E* and the underlying cortical nuclear layer at *D*. ×61,000.

Figs. 4–5.—Sections cut tangential to the nuclear surface. Labels in Fig. 4 also apply to Fig. 5. The difference in size of the pores is due to the different levels of section across them. Since the pores are cone-shaped the smaller annuli (*G*) are cut near the outer portion of the cortical layer or through the nuclear envelope and the larger ones (*F*) are sections through the pores near the inner surface of the cortical layer. Note dense granule near center of certain pores (*H*). Fig. 4 ×40,000; Fig. 5 ×61,000.

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METABOLIC HETEROGENEITY OF MOUSE LIVER DESOXYRIBONUCLEIC ACID

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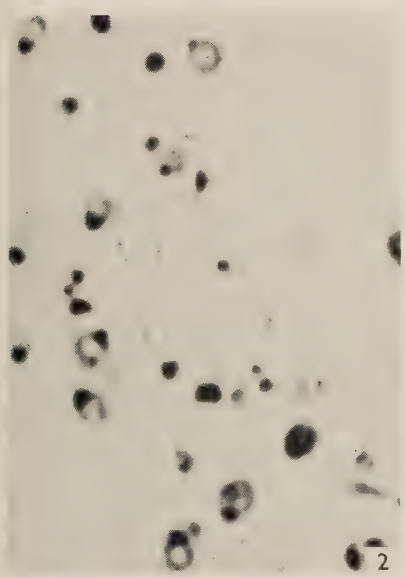
In a given tissue, the turnover of ribonucleic acids (RNA) occurs at a rate which depends upon the type of intra-cellular particle in which they are found: nucleus, mitochondria, microsomes, etc. These differences in metabolism of RNA related to differences in morphology have been studied by several authors who measured the incorporation of radiophosphorus (P^{32}) and of precursors marked with radiocarbon (C^{14}).

In the case of desoxyribonucleic acid (DNA), entirely localized in the cell nucleus, it is equally possible that metabolic differences exist that may be related to different morphological structures of the cell nucleus. The following work was carried out in order to study this point.

The experiments were carried out on genetically homogenous adult male mice (strain XVII of the Institut du Radium) in nutritional equilibrium with a complete diet. The mice received by sub-cutaneous injection 1 microcurie of P^{32} per gram of body weight in the form of Na_2HPO_4. The animals were sacrified exactly 1 hr. 15 min. and 5 hr. after the injection. The liver nuclei were isolated by the method published previously using solutions of polyvinyl pyrrolidone, a water-soluble resin (Subtosan) [7]. The nuclei were carefully purified by means of the method of superposition of layers of different density [6]. Their cytological behaviour was controlled

Fig. 1. (*a*) Entire nucleus with the nucleoplasm finely precipitated by the action of calcium ions present in the isolation medium. A detailed cytological study of the behaviour of nuclear structures during the isolation process has been presented in a preceding paper [7]. (*b*) Piece of an incompletely broken nucleus. (*c*) Large, free nucleoli with the associated chromatin in the form of large, compact lumps. This chromatin forms an integral part of the mouse liver nucleolus [7]. Some nucleoplasmic filaments can be noticed still attached to the body of the nucleolus (see Fig. 3). (*d*) Medium and small nucleoli showing the same characteristics as the large ones. Nucleoli which have lost their associated chromatin during breaking process. Free lumps of nucleolus associated chromatin. (*e*) Large filamentous debris; the nucleoplasm is precipitated by the calcium ions in the isolation medium before the nucleus is broken. It appears as an extremely fine filamentous structure whose meshes are filled with grains of scarcely visible chromatin swimming in a fairly clear liquid. After agitation in the mixer, the same more or less interlocking filamentous structure is found. (*f*) Small filamentous debris with nucleoplasmic chromatin dust.



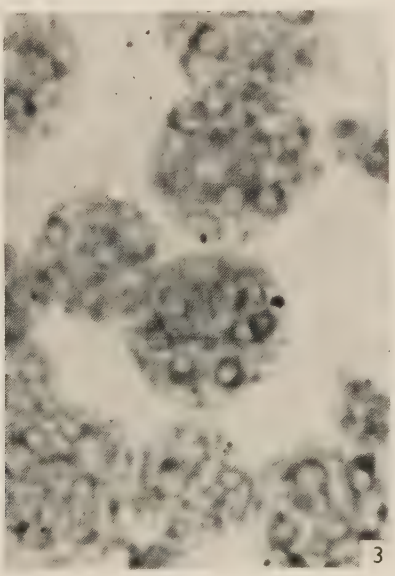


Fig. 2. Isolated liver nuclei stained by the Feulgen technique; the lumps of nucleolus-associated chromatin are strongly Feulgen positive.

Fig. 3. Feulgen stained smear of pellet I taken at the same magnification as Fig. 2. Free nucleoli of large, medium and small size, and some free lumps of nucleolus-associated chromatin are present.

Table I

Fraction	Time after injection of P^{32}	Spec. activity counts/min per 100 μg DNAP	Relative specific activity		
			Entire nucleus = 1	Nucleoplasm = 1	Nucleoli = 1
Entire nuclei	1 h 15 min	69[a]		1.6	0.94
	5 h	95		1.5	1
Nucleoli					
Pellet I	1 h 15 min	74	1.07	1.8	
(7 min, 1000 *g*)	5 h	100	1.05	1.5	
Pellet II	1 h 15 min	71	1.03	1.7	
(8 min, 8000 *g*)	5 h	91	0.96	1.4	
pellet I + pellet II =	1 h 15 min	73	1.07	1.7	
nucleoli *in toto*	5 h	96	1.0	1.5	
Nucleoplasm					
Pellet I	1 h 15 min	43	0.62	0.59	
(15 min, 15,000 *g*)	5 h	70	0.74		0.73
Pellet II	1 h 15 min	36	0.52	0.49	
(25 min, 21,000 *g*)	5 h	63	0.66		0.65
Supernate	1 h 15 min	46	0.67	0.63	
	5 h	64	0.64		0.66
pellet I + pellet II +	1 h 15 min	42	0.61	0.57	
supernate = nucleoplasm *in toto*	5 h	66	0.69		0.69

[a] The specific activities of the DNAP given in the table are the average of seven determinations and do not vary from corresponding highest and lowest observed values by more than 9 per cent.

throughout the preparation by examination in the phase contrast microscope and the calcium content of the isolation medium was maintained at a level which prevents any detectable swelling of the nucleole [7]. The nuclei, completely free of the cytoplasmic contaminations, were resuspended in a very viscous solution of dextran containing a small amount of calcium anions. The suspension is then agitated in a mixer. The various morphological elements thus freed from the burst nucleus are separated and collected by means of differential centrifugation in the cold. The identity of the various pellet and supernatants was verified by examination under the phase contrast microscope. A brief description of these various morphological elements is given in the legend of Fig. 1.

DNA of the different fractions is then isolated by the method of Schmidt and Thannhauser, modified according to McIndoe and Davidson [5], and the radioactivity of the phosphorus present in the DNA is determined.

The results are summarized in Table I.

The pellets I and II contain many large particles consisting mostly of free nucleoli. On the periphery of all these nucleoli there are many lumps of DNA-protein of regular shape, strongly Feulgen positive, and generally described as "nucleolus-associated chromatin" whose histochemical characteristics have been verified (Figs. 2 and 3). It can be seen that the incorporation of P^{32} in the DNA of these two fractions is more marked than in the sediments of nucleoplasmic origin.

The differences do not seem to be due to systematic contamination since the addition of an RNA carrier before the alkaline digestion and the use of a method employing lanthanum salts of nucleic acids after digestion of the nuclei by trypsin does not change the overall results.

It may be concluded that in the mouse liver nucleus in a condition of mitotic rest, the DNA turns over at a rate which varies according to the cytological localization of the DNA within the nucleus. These results are in agreement with the hypothesis of the heterogeneity of DNA within a given tissue which has been made by other workers [1, 2, 3, 4] and suggests that DNA may play a role in cellular metabolism. Research with precursors marked with radiocarbon is in course.

Summary.—Nuclei isolated from mouse liver and submitted to controlled breakage in a mixer are fragmented into particles which can be fractionated by the methods of differential centrifugation. The incorporation of radiophosphorus into the DNA of the various fractions differs significantly. The fractions rich in chromatin associated with the nucleolus show the highest specific activities.

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BIOPHYSICAL PROPERTIES OF THE TECTORIAL MEMBRANE (OF CORTI)

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Preliminary research by polarized light microscopy on histologic sections of the cochlea [5] has offered some suggestion as to the nature and structure of the tectorial membrane of Corti's organ.

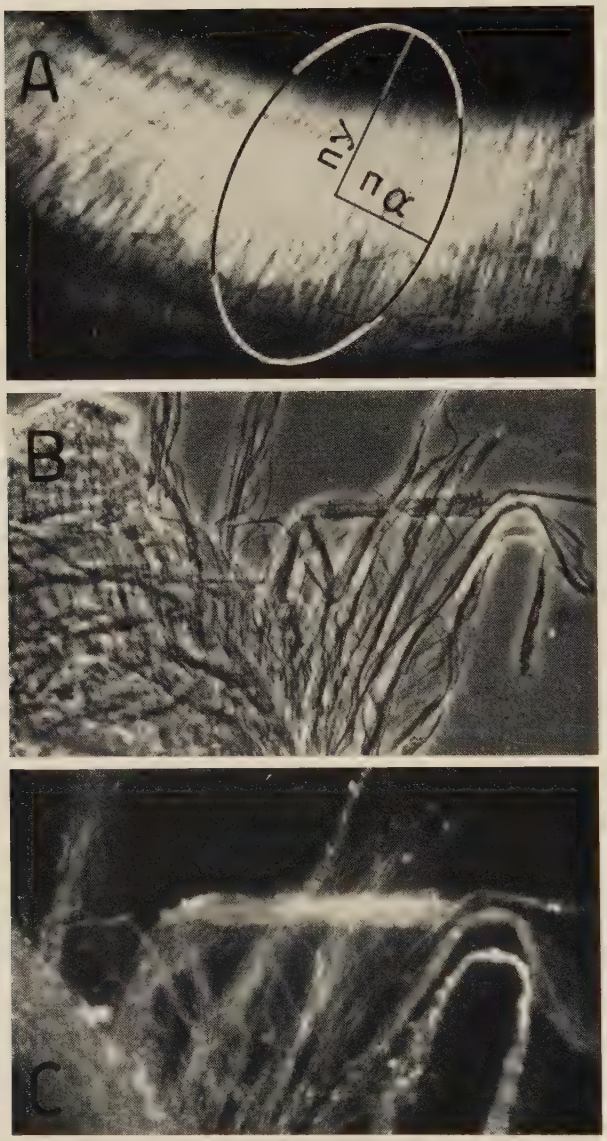
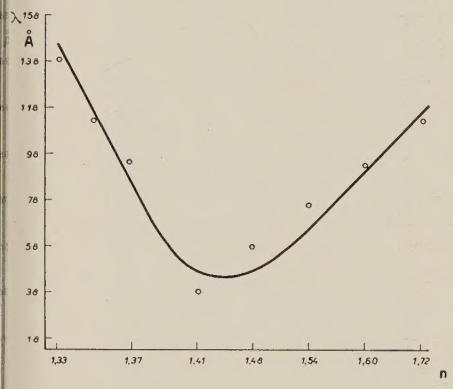


Fig. 1.—Tectorial membrane of *Mus musculus*. *A*, Polarized light microscope examination, × 350; *B*, phase contrast examination after partial dissociation of the tectorial membrane, × 800; *C*, the same fragment as in *B*, dark field microscope examination, × 1100.

Topochemical and enzymatic investigations pointed to the existence in the tectorial membrane of a protein having some of the optical features of fibrillar collagen: but this material is digested by neutral or alkaline trypsin and is slightly acid and base resistant.

By a special technique in preparing the cochlear duct [6] it was possible to isolate the tectorial membrane from the different whorls of the cochlea. Consequently,



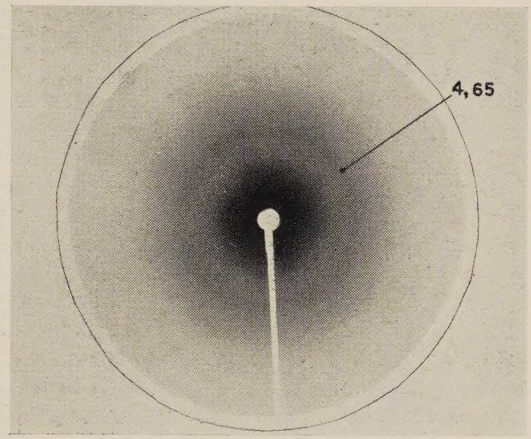


Fig. 2 (left).—Curve of retardation values of the tectorial membrane measured with the compensator of Brace after successive imbibition with liquids of different refraction index. (Potassium mercury iodide solutions).
Fig. 4 (right).—High angle X-ray diffraction diagram of tectorial membranes (specimen–plate distance = 50 mm, 3 hours' exposure, 35 KV, 13 MA).

better conditions were obtained for new microscopic and submicroscopic investigations on the tectorial membrane, spread out and examined by transparence. It was also possible to obtain specimens suitable for electron microscopy after fragmentation, high angle X-ray diffraction analysis and preliminary chemical investigation. The remarkable thinness of the tectorial membrane permits observations with the phase contrast microscope.

The principal part of the membrane reveals a coarse fibrous radial structure, while the end parts, one more in the centre towards the lamina spiralis, the other towards Hensen's cells, are thinner and have a trabecular appearance [7].

The fibrous structures are rather stiff, coarse and very closely packed the contours being difficult to define accurately. Treatment with trypsin carried out under microscopic control shows that all structures are digested and destroyed: no part of the tectorial membrane withstands this enzyme.

New observations in polarized light added further details to the data already reported by one of us [5]. The tectorial membrane has an intrinsic birefringence which is positive with reference to the rays radiating from the modiolus (Fig. 1 *A*) and is inverted by carbolic acid; it is modified neither by treatment with hyaluronidase nor by lipid extraction. Moreover, the tectorial membrane has a textural birefringence, suggesting that the submicroscopic organization is a prototype of a composite body: the diagram in Fig. 2 shows the variations of the textural birefringence as a function of the refraction index of the imbibing medium.

Dark field microscope research carried out on fragments of the membrane showed that the fibrous structures visible in phase contrast diffract light non-homogeneously: even the thinnest filaments have this appearance and no individual fibrillae are ever present (Fig. 1 *B*, *C*).

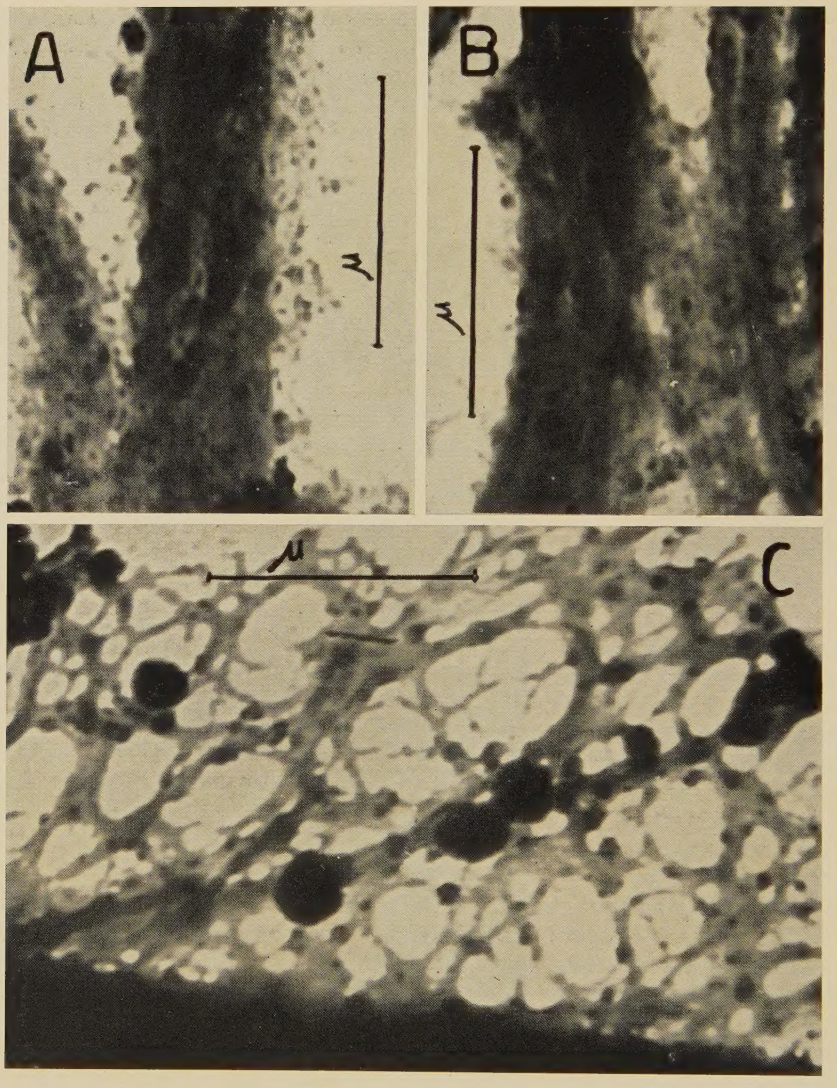


Fig. 3.—Tectorial membrane of *Mus musculus;* osmic fixation and fragmentation. *A*, *B*, Principal part of the tectorial membrane; Electr. Micr. Siemens Elmiskop I. *C*, End part overlapping Hensen's cells. Electr. Micr. Siemens Elmiskop I.

Osmic acid fixed membranes were fragmented in order to obtain specimens suitable for electron microscopy. The resulting micrographs are shown in Fig. 3. The coarser filamentous parts, seen under the light microscope, may easily be identified; the lack of true submicroscopic individual fibrillae is ascertained; the most

unravelled fibrillae show an end-to-end orientation which is also visible in some small irregularly placed globules (Fig. 3*A*, *B*). Consequently the electron microscope did not reveal the existence of submicroscopic collagen fibrillae [10, 12, 2]. The parts inserted either on Huschke's teeth or on Hensen's cells reveal the reticulated structure as shown in Fig. 3*C*; it has never been possible to see a submicroscopic periodic organization in the elements of the reticulum.

The hypothesis that there may be a cementing substance which could mask this supposed fibrillar structure, has prompted us to make several attempts with the aim at removing this assumed substance. These attempts (short tryptic treatment at different pH, acid or alkaline treatment) were unsuccessful.

The high angle X-ray diffraction analysis was made with the Cristalloflex III Siemens apparatus on five hundred isolated and dry tectorial membranes [8]. The resulting X-ray diagram is shown in Fig. 4. The diffraction bands of collagen are not visible; the only clear diffraction ring corresponds to a typical distance of 4.65 Å, which is rather characteristic of the K.E.M.F. proteins, in beta phase.

Table I shows the results of the chemical investigations which we could perform [8].

TABLE I

Amino acid composition of the tectorial membrane.
Grams per 100 g protein: quantitative paper chromatography after Pernis and Wunderly [9].

Glycine	5.7	Serine	4.1
Alanine	5.8	Threonine	3.6
Valine	4.9	Lysine	6.8
Leucine + Isoleu.	10.1	Tyrosine	2.9
Aspartic acid	8.2	Phenylalanine	4.2
Glutamic acid	10.2	Hydroxyproline[1]	0.0

Carbohydrates of the tectorial membrane.
(Methods by Dische [4]).

Hexoses	Hexosamines	Hexuronic acids
absent	about 1 per cent	absent

[1] Hydroxyproline has been determined by the method of Troll and Cannan [11].

It appears that the tectorial membrane does not contain any hexuronic acid, while it contains some glucosamine; anyhow it is evident that it is mainly constituted by a protein. The quantitative aminoacid analysis is in accord with the X-ray diagram, inasmuch as there is no finding supporting the presence of collagen-like structures in the membrane; indeed the amino acid typical of collagen (hydroxyproline) is absent in the proteins of the membrane, glycine is also present in comparatively low amount.

Conclusions.—The light and electron microscopic results had suggested that the tectorial membrane might be considered as a cuticular structure of the type of the

secreted collagens with no fibrillar organization; it would then be compared with the crystalloid, a well-known example of laminar but not fibrillar collagen [1].

However, the X-ray and chemical investigations do not confirm this hypothesis as they show that the proteic element must be considered to belong to the K.E.M.F. group.

These results suggest that (*a*) only chemical and X-ray investigation can define the nature of a given histological object; some microscopic tests, considered till now as significative in order to identify some proteic structures are, on the contrary, non-specific: (*b*) the tectorial membrane, cuticle of an epithelial and ectodermic origin shows some characteristics which rather approach it to the epidermal proteins and to the filamentous protein of the glia [3].

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